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EXPERIMENTAL EVOLUTIONARY BIOLOGY OF LEARNING IN *DROSOPHILA MELANOGASTER*

THESE

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Abstract

Many animals, including small invertebrates, have the capacity to modify their behaviour based on experience – they can learn. The ability to learn is itself a product of evolution, and the degree to which a given behaviour is modified by learning varies among species and among different types of behaviour. For an evolutionary biologist this raises the question of the conditions under which learning ability should evolve. The extent to which natural selection favours investment in developing learning ability will depend on the balance between costs and benefits of learning. On the one hand, it has been proposed that learning should be advantageous when the environment is temporally or spatially variable. On the other hand, constitutive or induced costs of learning have been repeatedly postulated and may counter select its evolution.

The general aim of this PhD is to understand under which environmental conditions learning ability should evolve, what are the costs of learning ability and how an opportunity to learn influences the evolution of innate behaviour. To address these questions I carried out experimental work with *Drosophila melanogaster*. Under an experimental evolution set-up, I selected flies with higher learning ability and showed the adaptive value of learning under variable environment. However, lines selected for improved learning abilities pay constitutive and induced fitness costs associated with increased learning ability. I also present experimental evidence that learning ability can evolve under constant environment and accelerates or slows down the evolution of innate response. I investigated this effect with mathematical simulations.

By decoupling the behaviour into its innate and learned component, this work provides evidence about the condition of evolution of each component and the evolutionary interaction between them.

Résumé

La plupart des animaux, invertébrés inclus, ont la capacité de modifier leur comportement à la suite d'expériences, ils sont capables d'apprendre. Leur capacité d'apprentissage est elle-même un produit de l'évolution et le degré de modification d'un comportement par l'apprentissage varie selon les espèces et selon le comportement considéré. Pour un biologiste de l'évolution ceci soulève la question des conditions sous lesquelles les capacités d'apprentissage devraient évoluer. La façon selon laquelle la sélection naturelle favorise l'évolution des capacités d'apprentissage doit dépendre de la balance entre coûts et bénéfices liés à cette capacité. L'argument général est que l'apprentissage devrait être favorisé dans des environnements à fortes variations temporelles ou spatiales. En revanche, l'existence de coûts constitutifs ou induits pouvant contrecarrer cet avantage a été plusieurs fois postulé.

Le thème général de cette thèse est l'étude des conditions environnementales sous lesquelles les capacités d'apprentissage devraient évoluer, des coûts et bénéfices associés à ces capacités et de l'impact de l'apprentissage dans l'évolution de la base innée du comportement. Afin de répondre à ces questions j'ai réalisé des expériences sur *Drosophila melanogaster*. Grâce à une approche d'évolution expérimentale j'ai sélectionné des drosophiles ayant une plus grande capacité d'apprentissage mettant ainsi en évidence la valeur adaptative de l'apprentissage en conditions environnementales variables. Cependant, ces drosophiles sélectionnées payent en retour un coût constitutif et un coût induit affectant leur fitness. Par un procédé similaire, je montre aussi que les capacités d'apprentissage peuvent évoluer dans un environnement constant et accélérer ou ralentir l'évolution des réponses innées. J'ai étudié cet effet en réalisant des simulations théoriques.

En découplant le comportement en sa composante innée et sa composante acquise, ce travail met en évidence les conditions d'évolution de chaque composante ainsi que les interactions entre elles et la conséquence de ces interactions sur leur évolution respective.

Introduction

"L'insecte aurait acquis son savoir-faire petit à petit, d'une génération à la suivante, par une longue suite d'essais fortuits, de tâtonements aveugles? Si c'etait là cocasserie de table après boire, entre la poire et le fromage, volontiers, je ferais chorus ; mais hélas! trois fois hélas cela se debite sans rire, magistralement solanellement, comme le dernier mot de la science "
(Fabre JH, souvenirs entomologiques, 1879)

During the antiquity and the middle age animal behaviour was seen with a very strong anthropomorphic view. Like humans, animals were considered to have learning abilities, memory, even intelligence and consciousness. The praying mantis was supposed to be a very religious animal whereas some populations of caterpillars had been sent to a court of justice for their devil behaviours (reported by Reaumur, 1734). This view of animal behaviour was completely rejected by Descartes for whom animals were just robots directed by God and had no intelligence or learning abilities at all (Descartes, 1617). For Descartes, learning could only occur if an animal would have a certain level of consciousness of itself. However, as animals do not have any conscious, animals are not learning. Descartes' view had a strong impact in all Europe during the seventeenth and the eighteenth century and some famous naturalists like Buffon often agreed with his theory (Buffon, 1749) despite some severe criticisms (Leroy, 1764).

The concept of instinct and learning and the abolishment of the "divine providence" origin of the behaviour were only defined in the beginning of the nineteenth century (Cuvier, Reaumur 1734, Romanes 1884, Gall 1812, Darwin 1871...). Learning was considered as a modification of the behaviour within lifetime due to experience whereas instinct was fixed. Two major questions were raised: the understanding of mental evolution and the transmission of behaviour from generations to generations (Lamarck 1809, Gall 1812, Cuvier, Darwin 1871).

<u>The understanding of mental evolution: from general process to adaptive process</u>

The existence of mental evolution was first exposed by a demonstration of intellectual continuity among animal species, including human. The observation of learning abilities differences among animals led first to the conclusion that learning has evolved following one universal mechanism. This 'general learning theory' considered the evolution of learning as a consequence of the global evolution of complexity in nature (Romanes 1884, Cuvier, Flourens 1851). Thorndike (Thorndike, 1911) declared that the main purpose of the experimental study of animal learning was "to learn the development of mental life down through the phylum". By that he meant that its aim should be to establish continuities in learning mechanism among species, showing how the capabilities of more advanced forms might have been derived in evolution from those of more primitive animals. Following this theory, primary studies have tried to rank animals in relation to their learning abilities (Gall 1812, Cuvier, Romanes 1884) and have tried to demonstrate the "directionality" of the evolution of mental abilities. These first works were mainly based on accumulation of anecdotes. It is therefore not surprising to see that domestic animals were considered as having better learning abilities than wild ones (with the notable exception of the orang-outang which was considered by Cuvier as one of the most "intelligent" animal for it strong docility). However, Thorndike proposed that controlled experimentation could offered the only hope for any real insight into the mechanism of learning, and his work opened the possibility of a direct comparison of learning abilities among animal species. This approach led

to the observation that different close related species could differ in performance on the same learning task. This finding did not contradict the theory of "general learning process": as learning is a "supraspecific" characteristic, difference in performance can be interpreted as difference in perceptual or motor capabilities.

At the beginning of the twenty century, ethology, the study of animal behaviour in relation to their environment, took off with the studies of Tinbergen and Lorenz on instinct and imprinting. Both criticized the theory of the "general learning process" (Tinbergen 1951, Lorenz 1965). Such criticisms have been followed by others in 1966 when Garcia and Koelling (Garcia and Koelling, 1966) discovered that rats were able to learn to avoid a sweet-tasting solution when its ingestion was associated with toxicosis but not when it was associated with foot shock. This finding was in conflict with the ideas of the general process of learning (Seligman, 1970). It showed the existence of some biological constraints on the animal learning abilities, constraints drawn by the evolutionary history of the species concerned. This result, combined with an increasing acceptance and understanding of Darwin theory of natural selection, led to the conclusion that animal learning abilities should be studied in terms of an animal overall biological adaptation to its environment.

It is therefore only recently that differences in learning ability among species and population began to be seen in the context of their natural environment and analysed in terms of benefits and fitness cost. This approach, often described as cognitive ecology (Real 1993), has witnessed a significant growth within the last couple of decades, both in terms of theory and empirical data (Dukas 1998; Shettleworth 1999; Johnston 1982; Papaj and Prokopy 1989; Healy and Braithwaite 2000). Closely related species, or even conspecific populations, often differ in their learning abilities in a way consistent with their ecological differences (e.g., Gould-Beierle and Kamil 1998; Girvan and Braithwaite 1998; Jackson and Carter 2001). Advances in theory and concepts have helped to integrate the evolutionary views of learning with the general evolutionary theory and identified general questions to be addressed by empirical research (Johnston 1982; Maynard-Smith 1987; Stephens 1991; Papaj 1994; Dukas 1998; West and Cunningham 2002).

Evolution of learning: a cost and benefit approach

The evolution of learning ability has therefore been proposed to be dependent on the interaction between an individual and its environment. A cost and benefit approach allows to make statements about the conditions under which learning abilities should evolve. In an environment, a phenotypic trait should evolve only if its total benefits outweigh its total cost.

The benefits of learning have been hypothesised since a long time. An organism that can learn obtains more information about its environment and hence can adapt more successfully to it. However, this statement implies that learning should always evolve independently of the environment in which evolution is occurring. The adaptive significance of learning can only be assessed with respect to particular environmental factors. The most widely accepted benefits of learning is that it allows an individual to adjust its behaviour in an adaptive way in a changing environment, where fitness consequences of a given action vary from generation to generation, or even within the lifetime (Johnston 1982; Papaj and Prokopy 1989; Stephens 1991; Dukas 1998). Modification of innate behavioural response can only occur at the population scale (via reproduction) and between generations whereas modification of behavioural response due to learning can occur at the individual scale within generation. Moreover, in contrast to phenotypic plasticity for which a predetermined phenotypic trait is produced in response to a predetermined environmental stimulus, learning has an additional level of flexibility, which allows an individual to change either the stimulus it responds to, or the response to the same stimulus (Dukas 1998). Some comparative studies among

close related species or populations of the same species have demonstrated a link between environmental stability and learning abilities (Girvan and Braithwaite, 1998; Gould Beierle and Kamil, 1998). Other benefits of learning have been proposed (Johnston, 1982): vocal learning in songbirds may enhance sexual selection; learning may compensate for innate lack of variation…

Several fitness costs of learning and memory have also been postulated (Johnston, 1982). The most obvious and well-described cost is the cost of being naïve: in order to master a specific task, an individual needs experiences composed of trials and errors. This might therefore require time, loss of energy intake and errors can be potentially risky for the individual. Examples include the practice time bumblebees need before they can handle a novel flower efficiently (Laverty and Plowright 1988), and mortality of bird fledglings due to inexperience in food gathering (Sullivan 1988; Johnston 1982; Dukas 1998). Costs could also be paid by individuals that exercise their learning ability. It concerns the energetic cost of collecting, processing and storing information during the learning process. Little is known about the magnitude of this cost. The process of learning has been shown to induce important neurobiological and morphological reorganization. Laughlin and co-workers (Laughlin et al. 1998, Laughlin 2001) have recently measured the metabolic cost of information processing in a photoreceptor of the fly compound eye. They showed that brains and sense organs use significant quantities of metabolic energy to process information. Such energy expense might represent a significant cost reflected in other fitness traits. Finally, costs of learning ability could be paid by individuals with genetically high learning ability whether or not they actually exercise this ability. They can be seen as pleiotropic effects of genes that improve learning ability. Such effects may be due to development and maintenance costs of the neural structures involved in learning and memory (Dukas 1999). For example, the size of the hippocampus (a vertebrate brain structure known to play a role in memory) has been found to be positively correlated with spatial learning ability among species (Biegler et al. 2001), among genotypes within species (Crusio and Schwegler 1993), and between the sexes (Jacobs and al. 1990). These structures are likely to be energetically costly (Sokoloff 1960; Dukas 1999). As natural populations face a limited world, this extra energy expenditure should be reflected in reduction of survival or fecundity: energy and proteins invested in the brain cannot be invested into eggs, somatic growth, or immune system. Hence learning ability is expected to show evolutionary trade-offs with some other fitness-related traits.

These proposed benefits and costs have mainly been studied by comparative studies among species or among population of the same species. If several studies have focused on the benefits, very few have focused on the costs of learning. It is particularly not known if the evolution of learning is constrained by some trade-off with other life history traits. The comparative approach has offered interesting insights and suggested novel hypotheses. However, since it studies the pattern rather than the evolutionary process leading to it, its inferences are indirect. It is not clear whether the cost and benefits observed have a direct effect on the fitness of the individuals. Comparisons between species are also problematic as phylogenetic differences could be an important confounding factor even if corrections are possible. Moreover, the comparisons have usually been done using data describing different behaviours, which might also affect the conclusions of these studies.

Transmission of the behaviour from generations to generations

The understanding of the diversity and complexity of the behaviours in the animal kingdom and their transmission from generations to generations has been a central problem in evolutionary biology. For Aristotle, Thomas Aquinas and Descartes the behaviour of an animal was given by God and therefore could not be explained. The concept of “instinct through divine providence” has been abandoned in the beginning of the

nineteenth century and replaced by an “instinct caused by the environment” theory. Erasmus Darwin (grandfather of Charles) believed that all behaviour was based on the experience and intelligence of the individual organism, and described ways in which apparently instinctive behaviours could be explained as such. Lamarck formulated a materialistic account of how the habits of animals of one generation could be changed into the instincts of their descendants. According to Lamarck, changing environmental conditions forced organism to change their habits. These changed habits involved increase use of certain body structures and organ systems along with the decreased use of others, with resulting organic changes being passed on to succeeding generations. Since behaviour is clearly influenced by biological structures, the inheritance of such modified structures would result in the instinctive behaviour dependent on the structures in succeeding generations. In this way Lamarck provided an explanation for the origin and the transmission of instinctive behaviour (Lamarck 1809). This theory had the advantage of avoiding a supernatural creator and the belief that acquired characteristics were inherited by one’s offspring was well accepted in Europe throughout most of the nineteenth century. Charles Darwin also first believed that beneficial habits that persisted over many generations would make heritable changes in the organism leading to instinctive behaviour in latter generations (Darwin 1872). He however became dissatisfied with this idea as the sole explanation for instinctive behaviour particularly when he realized that many of these behaviours could not have originated as habits. A major example discussed by several authors was the understanding of insect oviposition behaviour: How can an insect learn how and where to oviposit? Adult insect have usually never met their parents, never met their offspring and lay once eggs on a medium that they do not usually use (Romanes, 1884; Fabre, 1879). Darwin’s theory of selection applied to animal behaviour proposed therefore an opportunity for natural selection on instinct to work: the evolution of instinct does not require any form of learning but natural selection plays on the diversity of innate response within populations.

Despite the enormous impact that Darwin had on the life sciences during his own lifetime, he had relatively little immediate impact on the scientific study of animal behaviour. This was mainly due to the methodological difficulties and the extensive use of anecdotical evidence and anthropomorphic interpretation (Romanes, 1884). Konrad Lorenz was primarily interested in finding evolutionary explanations for instinctive behaviours (Lorenz, 1965). He posited a genetic basis for specific behaviours that was subjected to the same principles of cumulative variation and selection that underlie complexity of biological structures.

However, the general dichotomous view of behaviour as either mostly learned or mostly innate ignores the importance of the interaction between the evolution of the innate component and the evolution of learning ability. Behaviour is the result of a complex interaction between a learning component and an innate one and, if natural selection can act directly on the behaviour, it can only act on an indirect way on each component. In order to understand the evolution of instinct, it is therefore important to understand how learning can affect this evolution. On the one hand, learning may allow an individual to behave optimally irrespective of its genotypic value for the innate component of the behaviour. Thus, the ability to learn partially decouples the behavioural phenotype from the genotype. It has been proposed that this effect should make natural selection on the innate, heritable component of the behavioural_response less effective, slowing down its evolution (Papaj 1994; Robinson and Dukas 1999). In contrast, a simulation model by Hinton and Nowlan (1987) suggested that in a novel environment learning might accelerate the evolution of the innate component towards the optimum. A similar prediction has been obtained in artificial intelligence models (Belew 1989; Ackley and Littman 1991; French and Mesinger 1994; Mayley 1997). These models provide some formal underpinning for the old verbal arguments that learning may accelerate evolution (Baldwin 1896; Morgan 1896; Osborn 1896). However, quantitative genetic models making

more general assumptions about the fitness function (Anderson 1995; Ancel 2000) suggested that the conditions under which learning may facilitate evolution are highly restricted. The understanding of the evolutionary relationship between innate and learned component of the behaviour is fundamental for a better understanding of the evolution of the behaviour.

DESCRIPTION OF THE THESIS

Within the last four years I developed a research program centred on the evolutionary biology of associative learning ability in Drosophila. In addition to the usual advantages (genetic tools, short generation time), D. melanogaster is capable of many different forms of learning and has at least four different types of memory. Within recent years it has become a favourite model organism for neurobiological research on learning.

We decided to concentrate on the role of associative learning in oviposition substrate choice. The choice of a suitable oviposition substrate is an ecologically important decision with a direct impact on fitness. It may be modified by experience because in nature Drosophila females lay eggs over extended time, potentially on many different substrates, which are also fed on by the adults. They can thus get a feedback (reinforcement) concerning the quality of the oviposition medium. These features open an opportunity for learning to contribute to Darwinian fitness under natural conditions and make it easy to create conditions favouring learning in the laboratory.

Chapter 1: Experimental evolution of learning ability in fruit flies

Several researchers successfully applied direct artificial selection to a particular conditioned behaviour, producing flies or rats with higher learning ability (Tryon, 1940; Lofdahl et al., 1992). These experiments demonstrate the existence of genetic variation for learning ability and its potential to evolve. Nevertheless, the direct artificial selection approach makes improved learning ability to be favoured irrespective of the costs. As I presented in the previous chapter, learning should be adaptive in a variable environment. However the evolution of learning under this conditions may be constraint by its potential costs (See Appendix 1). To mimic the setting under which learning may evolve in nature, I developed an experimental design that created ecological conditions under which learning ability in *Drosophila melanogaster* was supposed to evolve. In this approach, called experimental evolution, the contribution of learning to reproductive success is indirect and has to be set against its potential costs. This approach proved highly successful when applied to the evolution of senescence (e.g., Rose 1984; Zwaan et al. 1995), life history traits (e.g., Mueller and Sweet 1986; Hillesheim and Stearns 1992), or phenotypic plasticity (e.g., Scheiner and Lyman 1991). It allows one to observe the evolutionary process directly while controlling for confounding factors.

After 23 generations under this selection regime the experimental flies evolved markedly higher learning ability. I thus showed that learning might evolve in a laboratory setting under conditions mimicking natural selection. Other tests showed that these experimental flies evolved higher learning rate and improved memory but did not evolve higher discrimination ability or quinine susceptibility.

Chapter 2: A fitness cost of learning ability in *Drosophila melanogaster*

Costs of learning ability have been rarely studied. However, the understanding of learning evolution needs the study of both the benefits and the cost associated to learning ability.

In artificial selection, individuals carrying the selected phenotypic trait will be selected to form the next generation. As long as the cost induced by this phenotypic trait or by other traits evolving simultaneously by linkage are not lethal, and as long as there is genetic diversity for the trait artificial selection can occur. Consequently, the study of fitness cost associated to the evolution of the trait might be misleading by using this type of selection. The correlated costs might be overestimate as they will be partly due to the evolution of the focused trait but also partly due to the evolution of the traits evolved by linkage. The experimental evolution approach may partially solve this problem. In experimental evolution a trait will evolve only if its benefits outweigh its costs. Therefore one expect using this type of selection, linked deleterious traits should be counter-selected and that the trait with the highest benefits over cost ratio should be selected. Following this argument, experimental evolution offers a good opportunity to measure the fitness cost of the evolved trait

In this experiment, I proposed to study the potential constitutive cost of learning ability. Learning ability is unlikely to be cost-free. Information processing and storing, as well as development and maintenance of neural structures involved in learning and memory, are energetically costly. As in nature resources are limited, this additional energy expenditure should be reflected in reduction of survival or fecundity: energy and proteins invested in the brain cannot be invested into eggs, somatic growth, or immune system. Hence learning ability is expected to show evolutionary trade-offs with other fitness-related traits but such trade-offs had not been experimentally demonstrated. I showed that experimental flies, selected for improved learning ability, had significant lower larval competitive ability than control flies. I interpret the reduced larval competitive ability of the experimental lines as a genetic cost of having evolved higher learning ability; this cost would be paid irrespective of whether a given fly ever learns anything.

Chapter 3: An induced fitness cost of learning in *Drosophila melanogaster*

In this experiment I focused on a different type of cost which is cost paid by an individual which is exercising its learning ability. As the learning and memory process requires energy, one might expect that individuals which often learn during their life would spend more energy than the same individuals under conditions where they do not learn. This difference of energy demand could be reflected in a reduction of another fitness-related trait. Following this hypothesis, I conducted an experiment in which experimental and control flies were placed under two different life treatments: in one flies were daily faced with a learning task whereas in the other one flies were kept under similar conditions but had nothing to learn. The results indicated that repeated learning events decrease significantly the fecundity and the learning ability of the flies. We observed a trade off between learning ability and fecundity. These results may indicate that, if learning is advantageous under variable conditions, too frequent modifications of the environment within the lifespan of the individual might counter select its evolution.

Taken altogether, these two last experiments clearly indicate that learning has fitness costs. According to the adaptive theory of the evolution of learning, these results support the hypothesis that learning should only evolve under temporally or spatially heterogeneous environment, when it benefits outweigh its costs

Chapter 4: Evolutionary interactions between the innate and learned components of behavior: experimental evolution of oviposition substrate preference in *Drosophila melanogaster*

If learning ability presents fitness cost one should not expect the evolution of learning ability under stable environmental conditions but only evolution of innate response. I tested this assumption using a similar

experimental evolution design. I observed that even when the environmental conditions were stable learning abilities evolved. Therefore, when both learning and innate response are under selection, learning can still be beneficial despite its fitness costs. Furthermore, the evolution of learning ability affects the evolution of innate response. I show experimentally that evolution of learning can either slow down or accelerate the evolution of innate response. I propose that the effect of learning on evolution of innate response depends on the way learning modifies the selection differential: when learning increases the selection differential it accelerates evolution, whereas when learning decreases this selection differential, it slows down evolution.

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CHAPTER 1: Experimental Evolution of Learning Ability in Fruit Flies

The presence of genetic variation for learning ability in animals opens the way for experiments asking how and under what ecological circumstances improved learning ability should evolve. Here we report experimental evolution of learning ability in *Drosophila melanogaster*. We exposed experimental populations for 51 generations to conditions that we expected to favor associative learning with regard to oviposition substrate choice. Flies that learned to associate a chemical cue (quinine) with a particular substrate, and still avoided this substrate several hours after the cue had been removed, were expected to contribute more alleles to the next generation. From about generation 15 on the experimental populations showed marked ability to avoid oviposition substrates which several hours earlier had contained the chemical cue. The improved response to conditioning was also expressed when the flies were faced with a choice of novel media. We demonstrate that these behavioural changes are due to the evolution of both a higher learning rate and a better memory.

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Learning ability is known to respond readily to direct artificial selection on a particular conditioned behavior (1-5). In such experiments the conditionability of the focal behavior is the sole criterion that determines if an individual is allowed to breed. However, in natural populations learning and memory may entail fitness costs, if only due to energy needed to maintain neuronal information and underlying structures (6). It remains unclear how readily learning evolves under natural selection, when its contribution to reproductive success is indirect and has to be set against its potential costs (7-12).

To address this issue, we kept populations of *Drosophila melanogaster* under ecological conditions that we expected to favor the evolution of learning ability in the context of oviposition substrate choice. The choice of a suitable oviposition substrate is an ecologically important decision with a direct impact on fitness. It may be modified by experience because in nature *Drosophila* females lay eggs over extended time, potentially on many different substrates, which are also fed on by the adults. They can thus assess the quality of the oviposition medium, which, together with relatively well developed associative memory (13), opens an opportunity for learning to contribute to Darwinian fitness (11,12,14).

In our experiment the flies had a choice between two oviposition media, one of which had previously contained an aversive chemical (gustatory) cue. An individual would increase its contribution to the next generation by laying a greater number of eggs on the medium that had not contained the cue. This could be achieved in two ways. First, the individual could increase the total *number* of eggs laid within the oviposition period on both media, independently of the cue. This change would potentially be counteracted by tradeoffs such as reduced offspring quality. Second, a better ability to associate a medium with the aversive cue (i.e., better learning, better memory, and/or a better ability to discriminate between the media) would enable the individual to lay a greater *proportion* of eggs on the appropriate medium. These changes would not be favored if they entailed a sufficient cost in terms of reduced fecundity, such that the actual number of eggs laid on the appropriate medium would not increase. Thus, in contrast to artificial selection, under which a learning score is the only criterion used to decide if an animal is allowed to breed, our design allows processes other than learning to increase an individual's contribution to the next generation. How the populations would respond was likely to depend on the available genetic variation and the balance between costs and benefits. Our experimental populations did evolve a marked ability to modify their oviposition substrate preference in response to conditioning. We have been able to show that they achieved this by evolving both faster learning and improved memory but not by any detectable improvement of discrimination ability.

Material and Methods

Stock. Our base stock population of *Drosophila melanogaster* was derived from 2000 flies caught in Basel (Switzerland) and maintained for 6 months in the laboratory prior to the beginning of the experiment. All flies used during the experiments were 14 days old (counted from egg).

Experimental evolution design. Every generation 150 adult flies from each of eight experimental and eight control populations were transferred to cages (19 × 12 ×13 cm) and allowed to oviposit during three consecutive periods of three hours (Fig. 1) in complete darkness, 25°C and 70 % relative humidity. During each period we offered the flies a choice between two oviposition substrates: an orange medium and a pineapple medium. These media were prepared from 100% orange or pineapple juice from concentrate with 6.6 g/l agar added. At the bottom of the cage one petri dish with 10 ml of the orange medium and one with 10 ml of the pineapple medium were

attached at the end of plastic tubes (height 5 cm, diameter 6 cm). A fresh set of petri dishes with the media was provided at the beginning of each period; their position was randomized.

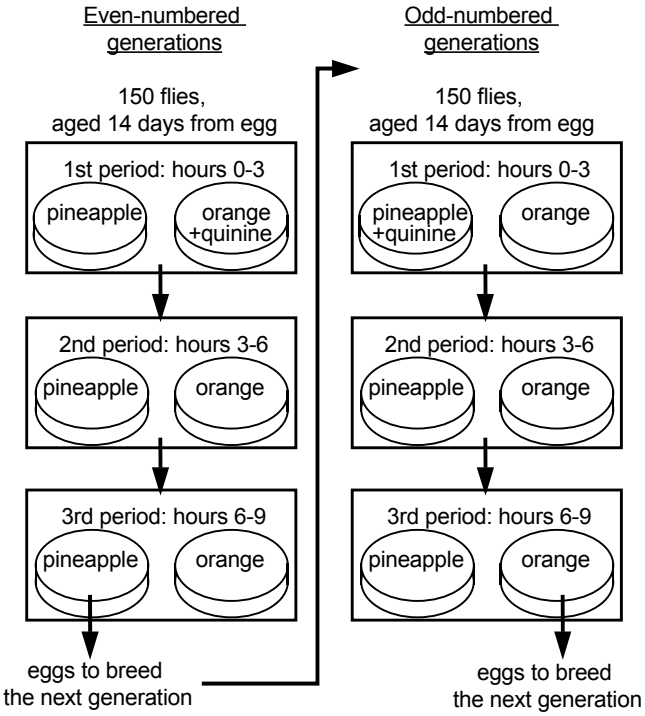

Figure 1: Design of the experimental evolution: selection regime in the experimental lines at even and odd-numbered generations. Only eggs laid in period 3 on one medium (orange in odd and pineapple in even generations) were used to breed the next generation. The regime experienced by the control lines was identical except that quinine was never added to any medium.

One of the media offered to the experimental populations in period 1 (alternately, pineapple in odd-numbered generations, orange in even-numbered generations) additionally contained quinine hydrochloride (4 g/l). At this concentration quinine in the fruit medium had no effect on fly fecundity (F. Mery, unpublished data). From the first generation the flies showed a strong avoidance of quinine: only 1.8 ± 0.9 % of the eggs were laid on the quinine-containing medium. This avoidance did not change throughout the experiment, indicating that the results we describe below are not due to improved quinine recognition. During this first period (the training period) flies from the experimental populations were thus expected to associate one of the two media with the presence of quinine. Quinine was not added to any medium offered during periods 2 and 3. The choice of oviposition medium in periods 2 and 3 (test periods) is therefore expected to reflect the conditioning that occurred in period 1. Two test periods were used to assess decay of the conditioned response with time (due to forgetting or extinction). Oviposition preference was scored as the proportion of eggs laid on each medium. The next generation was bred from 250 eggs laid in period 3 on the medium that had not contained quinine in period 1 (i.e., orange in odd-numbered, pineapple in even-numbered generations; see figure 1). These eggs were rinsed with water and transferred to a 250-ml bottle containing 21 ml of a standard cornmeal medium. Larvae were thus always reared on the same medium, which precluded any preferences induced by larval medium. The control flies were treated in the same way except that they were never given any medium containing quinine.

Experimental flies could thus learn to use the presence of quinine in period 1 as a cue indicating which medium they should avoid for oviposition in period 3, when no cue was present. The cue was not available to the control flies.

The experiment has now been running for 57 generations. Due to technical problems (accidental insecticide poisoning in the laboratory) at generation 27 one experimental (line 4) and one control line (line 3) were lost while the population size of some other lines was temporarily reduced (in one case to only about 20 adults). To facilitate recovery, selection regime was suspended for generations 27-31; selection was also not applied at generations 11, 35 and 44 for other reasons. At those generations flies laid eggs on a standard cornmeal medium.

Response to conditioning. At generations 23 and 46 we simultaneously assayed the effect of conditioning on the oviposition preference of flies from each experimental and control line, as well as from the stock population (kept on a standard cornmeal medium). Each line was divided in three samples. The flies of each group were introduced into cages and, as before, allowed to oviposit for 3 periods of 3 hours each. One sample was tested without conditioning (quinine not present in any medium), another sample with conditioning to avoid pineapple (quinine present in the pineapple medium in period 1), and the last sample with conditioning to avoid orange (quinine present in the orange medium in period 1).

At generation 43 we also tested the learning ability of all control and experimental lines when faced with two novel fruit media. These were an apple medium and a tomato medium, both prepared from juice and agar. This test employed the same design as described above.

Comparison of the rate of learning. The aim of this assay, performed at generation 47, was to obtain information about the time-course of the learning process, i.e., to see how the conditioned response depends on the amount (length) of conditioning. In order to be better able to control the time spent on a medium with quinine (i.e., the effective conditioning time), we used another conditioning regime. This conditioning regime, in contrast to that described above, did not allow the flies to switch freely between the quinine-containing and quinine-free medium. It involved cycles of a 45-minute "resting" period, during which the flies were kept in an empty vial, followed by a 45-minute conditioning period, during which the flies were kept in a vial with a quinine-containing orange or pineapple medium (quinine hydrochloride 4 g/l). The treatments consisted of exposure to one, three, five or seven such consecutive resting-conditioning cycles (corresponding to 45, 135, 225 and 315 min of total conditioning time). Prior to the first resting-conditioning cycle the flies used in the treatments with one, three and five cycles were maintained in empty vials for additional 540, 360 and 180 minutes, respectively. That way the total time spent during the conditioning phase in empty vials plus in vials with quinine-containing medium was the same in all treatments (630 min). Therefore, when their conditioned response was assayed, all flies had been prevented from egg laying for the same length of time and their motivation to oviposit should be similar (flies oviposit neither in a quinine-containing medium, nor in empty vials). All cycles of a given treatment involved conditioning to avoid the same medium (either orange or pineapple). In other words, during conditioning the flies repeatedly encountered either quinine-containing orange medium or quinine-containing pineapple medium, but no medium without quinine. There were thus eight treatment combinations (four durations of conditioning $\times$ orange or pineapple medium). Each of these eight treatment combinations was applied to a different sample of 50 flies (males + females) from each of five randomly selected experimental and five control lines (control lines 1, 2, 4, 5 and 7; experimental lines 1, 3, 5, 6 and 8). All fly transfers were done without anesthesia. The response to conditioning was assessed immediately

following the last resting-conditioning cycle. Each sample of flies was allowed to oviposit for 1 hour in a cage with one petri dish of the orange medium and one of the pineapple medium, neither containing quinine. The proportion of eggs laid on each medium was scored.

Decay of the conditioned response. The aim of this assay (done at generation 48) was to study how the conditioned response diminishes with time elapsed since conditioning. Differences in the rate of this decay between the experimental and control populations would suggest evolved differences in their memory. We used the same five experimental and five control lines as in the assay described in the preceding paragraph. We also used the same type of conditioning as described in the preceding paragraph, except that all flies were exposed to the same amount of conditioning: five resting-conditioning cycles. We knew from a previous assay that after five resting-conditioning cycles experimental and control lines show a similar conditioned response if tested immediately after conditioning (see Results). Four samples of 50 flies from each line were conditioned that way to avoid orange. The oviposition preference of one of the four samples was tested immediately following the last learning-conditioning cycle; the remaining three samples were transferred for 1, 2 and 3 hours, respectively, to empty vials ("forgetting period") before being tested. Another set of four samples of 50 flies were conditioned in an analogous way to avoid pineapple and tested in the same way. The oviposition preference of all eight samples was assessed by allowing them to oviposit for 1 hour in cages with one petri dish of orange and one of pineapple medium, both without quinine.

Dose-response curve. The aim of this assay (done at generation 56) was to test if salience of the pineapple and orange medium to elicit oviposition is greater in the experimental than control populations. We again used the same subset of five experimental and control lines. 100 flies from each line were presented for 3 hours with one petri dish with a pure agar medium (7g/l) and one with a diluted fruit medium. The fruit medium was composed of agar (7g/l) and orange or pineapple fruit juice diluted with water. We tested the following dilution series: 1 (pure juice), 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256. As flies refrain from laying eggs on a pure agar medium, the number of eggs laid on the fruit medium of a given concentration reflects the ability of the flies to detect this concentration and its salience to stimulate oviposition. To compare the dose-response curves of experimental and control populations we fitted a Poisson regression of the number of eggs laid on the fruit medium on $-\log_2$(concentration), with treatment (experimental versus control) as a main effect, and treatment × $-\log_2$(concentration) interaction testing for a difference of the slope. We also tested if differences among the five experimental lines in the response to declining medium concentration were correlated with differences in their learning ability. To this end we calculated Pearson's correlation between the coefficients of Poisson regression fitted separately to each line, and a learning score. The learning score for each line was estimated as a differences between between the proportion of eggs laid on orange when conditioned to avoid pineapple and when conditioned to avoid orange, assayed at the same generation as the dose-response curve (but using different individuals). The conditioning involved six resting-conditioning cycles of the type described in the subsection "Comparison of the rate of learning".

Analysis. SPSS statistical package was used for all analyses except for the Poisson regression, which was done with PROC GENMOD of SAS statistical software. The analysis treated the proportions of eggs laid on the orange medium by each line in each period as raw data. Because all proportion values fell in the range 0.30 – 0.84, they were not transformed before the analysis (15). Where the data from periods 2 and 3 were analyzed together, repeated measures analysis of variance was used, with line treated as the subject and period as the within-subject effect. Where appropriate, the residuals were checked for normality with the Shapiro-Wilk test. No deviations from normality were detected except in the case mentioned in the legend to Figure 2.

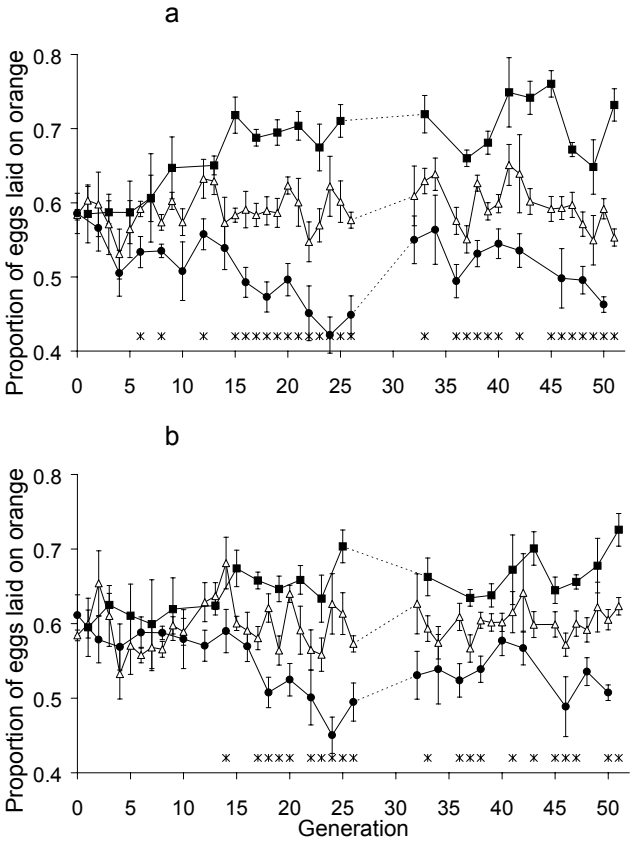

Figure 2: The proportion of eggs laid on the orange medium in the course of the experimental evolution (means ± standard errors). (a) period 2; (b) period 3. Circles: experimental populations at even-numbered generations (conditioned to avoid orange); squares: experimental populations at odd-numbered generations (conditioned to avoid pineapple); triangles: control populations (not conditioned). Data for generations 11, 27-31, 35 and 44 are missing. An asterisk indicates a significant difference (*t*-test, $p < 0.05$, not corrected for multiple comparisons) between the experimental and control lines. The data only deviated from normality at generations 32 and 45 ($0.05 > p > 0.01$).

RESULTS

Changes in the course of experimental evolution. Figure 2 shows the proportion of eggs laid on the orange medium by the experimental and control lines in the course of the experiment. At the beginning (generation 0) the proportion of eggs laid on each medium during periods 2 and 3 was not affected by the presence of quinine during period 1 (repeated measures analysis of variance, $F_{1,16} = 0.06$, $p > 0.8$). After several generations of experimental evolution, the experimental populations began to show an effect of conditioning on oviposition substrate preference. They laid in periods 2 and 3 an increasing proportion of their eggs on the "correct" medium, i.e., the medium that had not contained quinine in period 1 (Fig. 2).

From generation 15 until selection was suspended at generation 27 the experimental flies consistently laid in period 2 a significantly greater proportion of their eggs on the "correct" medium than the control flies; for the third period this held from generation 17. After the selection regime had been resumed at generation 32 the difference was less pronounced at some generations, but still consistent. The increasing difference between the experimental and control lines with respect to the proportion of eggs laid on the "correct" medium (Fig. 3) illustrates the evolution of

improved learning ability. The difference was typically smaller in period 3 than in period 2 (paired *t*-test, $p = 0.008$), suggesting that with time the flies either tended to forget the association between quinine and the medium or learned that quinine was now absent from the medium (extinction). We did not observe any change of fecundity of the experimental or control flies over generations (regression analysis; control: $F = 0.04$, $p > 0.8$; experimental: $F = 0.67$, $p > 0.4$).

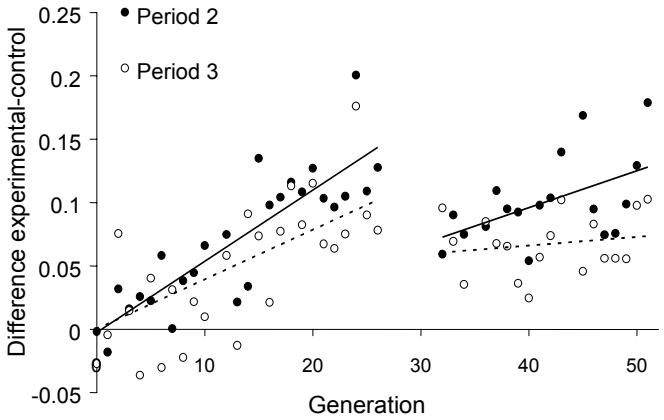

Figure 3: The difference between the experimental and control lines in the mean proportion of eggs laid on the "correct" medium (orange in odd-numbered, pineapple in even-numbered generations). Separate regression lines were fitted for the two testing periods (period 2 and period 3). Because selection was suspended at generations 27-31, regression lines for generations 1-26 and 32-51 were fitted separately; for generations 1-26 the lines were forced through the origin. For generations 1-26 both regression slopes are significantly positive ($p < 0.001$); after generation 32 only the regression for period 2 is significantly positive ($p < 0.01$).

Response to conditioning. The assays done at generation 23 and 46 showed that in the absence of conditioning the preference of the experimental flies did not differ from the control and stock (Fig. 4a; repeated measures ANOVA, $F_{2,24} = 0.19$, $p > 0.8$ and $F_{2,21} = 2.45$, $p = 0.11$ for generation 23 and 46, respectively). This indicates that no evolutionary changes of genetically based (innate) preference occurred during the experiment. However, the oviposition substrate preference of the experimental populations was strongly affected by conditioning ($F_{2,24} = 24.9$, $p < 0.001$ and $F_{2,21} = 41.1$ $p < 0.001$ for generation 23 and 46, respectively), whereas the control and stock flies showed no detectable response to conditioning (Fig 4a; generation 23: control: $F_{2,24} = 0.87$, $p > 0.4$; stock: $F_{2,24} = 1.42$, $p > 0.2$; generation 46: control: $F_{2,21} = 0.31$, $p > 0.7$; stock: $F_{2,21} = 0.72$, $p > 0.4$). There was no difference between the control and stock flies, indicating that the control flies did not evolve learning ability in the course of the experiment. For the experimental treatment we did not observe any difference of conditionability between generations 23 and 46.

When faced with two novel fruit media (apple and tomato), flies of the experimental lines still responded to conditioning (repeated measures ANOVA, $F_{2,21} = 5.09$, $p = 0.018$), whereas flies of the

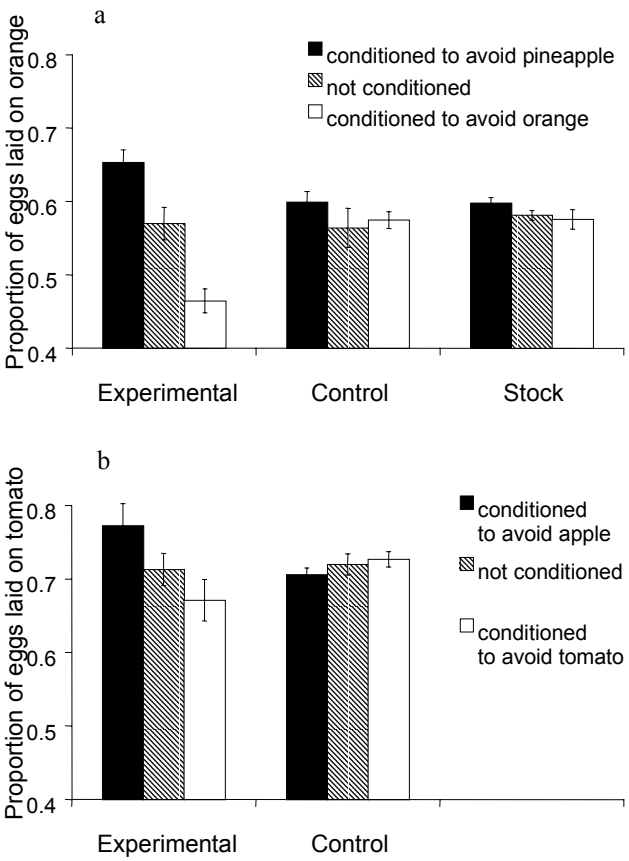

Figure 4: Response of oviposition substrate preference to conditioning, measured on two sets of media: (a) orange versus pineapple (generation 23); (b) apple versus tomato (generation 43). "Conditioned to avoid pineapple" means that quinine was present in the pineapple medium offered in period 1. The proportion of eggs laid on the orange medium was averaged over periods 2 and 3; bars represent standard errors.

control lines showed no response to conditioning (Fig. 4b; repeated measures ANOVA, $F_{2,21} = 0.82$, $p > 0.4$). The improved learning ability of the experimental populations was thus also manifested when they were faced with oviposition media other than those used in the course of selection.

Coson of the rate of learning (generation 47). As in the previous assay, in the absence of conditioning the oviposition substrate preference (the proportion of eggs laid on the orange medium) did not differ between the experimental and control flies ($p > 0.2$). The response of the oviposition substrate preference was a decelerating function of the total conditioning time (the number of resting-conditioning cycles), irrespective of whether the flies were conditioned to avoid orange or pineapple (fig. 5). Flies from the experimental populations responded to conditioning faster. They showed a significant change of their oviposition preference already after a single 45-minute conditioning event ($t = 3.01$, $p = 0.014$; $t = 5.11$, $p < 0.001$ for flies conditioned to avoid orange and pineapple, respectively). For the control flies the response was significant at $p < 0.05$ only after three (conditioned to avoid pineapple) or five (conditioned to avoid orange) resting-conditioning cycles, corresponding to a total conditioning time of 135 and 225 min, respectively. As a consequence, the experimental flies exposed to 45 and 135 min of total conditioning time showed a stronger response to conditioning than control flies, although for 45 min this difference was only marginally significant (fig. 5). However, when total conditioning time was longer, the response of the control flies became as large as that of experimental flies. After 315 min of conditioning the control flies showed almost exactly the same oviposition substrate preference as the experimental flies, both when

conditioned to avoid orange and when conditioned to avoid pineapple (fig 5). To summarize, although the control flies learned more slowly than the control flies (they showed a weaker response after a short conditioning time), their maximum response to conditioning seemed to be the same as that of the experimental flies.

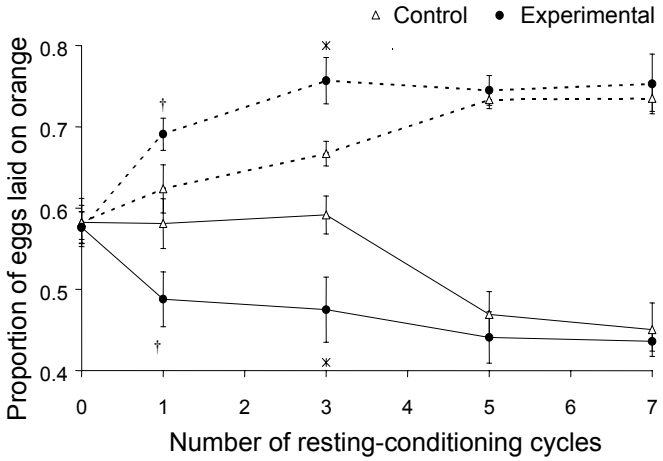

Figure 5: Comparison of the rate of learning (generation 47). The response of experimental and control populations to conditioning as a function of the total conditioning time (the number of resting-conditioning cycles). Solid lines: flies conditioned to avoid orange; dashed lines: flies conditioned to avoid pineapple. Bars represent ± one standard error; "*" indicates a significant difference (*t*-test, $p<0.05$) between the experimental and control lines for a given conditioning treatment, "†" indicates $0.05 < p < 0.08$.

Decay of the conditioned response (generation 48). The response of experimental and control flies to five cycles of resting and conditioning (total conditioning time 225 min) was statistically undistinguishable when tested immediately after conditioning (fig. 6). This result is consistent with the results from generation 47 described in the previous paragraph. In both experimental and control populations the effect of conditioning on oviposition site preference diminished with time elapsed since the end of conditioning, but this decay was faster in the controls (fig. 6). As a consequence, whenever there was a delay between conditioning and testing, the experimental flies laid a smaller proportion of eggs than the control flies on the medium they were conditioned to avoid. This held irrespective of the length of the delay (1, 2 or 3 hours) and of the medium they were conditioned to avoid (orange or pineapple). The experimental flies still showed a significant effect of conditioning if tested 3 hours after termination of conditioning ($t = 2.85$, $p = 0.011$ and $t = 6.68$, $p < 0.001$ for flies conditioned to avoid orange and pineapple, respectively). In contrast, no effect of conditioning could be detected in control flies already after 2 hours, nor after 3 hours. The faster decay of the conditioned response with time was also manifested in a significant interaction between time since conditioning and experimental versus control selection regime (two-way ANCOVA, $F = 5.27$, $p = 0.024$ and $F = 5.08$, $p = 0.026$, for flies conditioned to avoid orange and pineapple, respectively). This indicates that the experimental flies had a better memory than the controls.

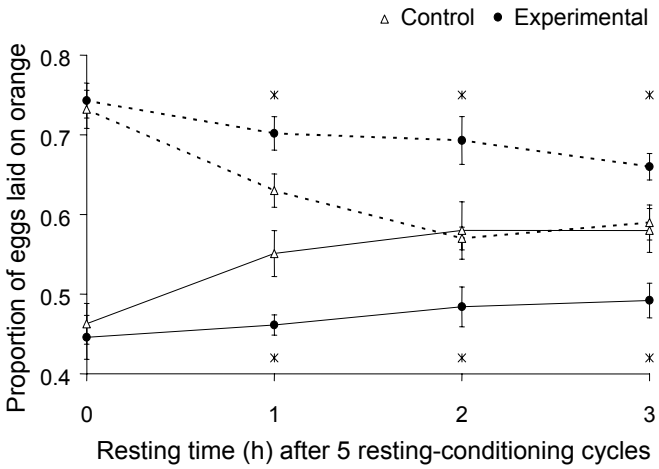

Figure 6. Decay of the conditioned response with time elapsed since the last conditioning cycle (generation 48). All flies were exposed to five resting-conditioning cycles, with total conditioning time of 225 min. Symbols as in figure 5.

Dose-response curve. The experimental and control lines responded similarly to decreasing concentration of juice in the medium, with a characteristic plateau at intermediate concentrations (Fig. 7). We detected no differences between the treatments in the intercept or slope of the Poisson regression of the number of eggs laid on the $-\log_2$(concentration) on either medium (all $p > 0.5$). The differences among the replicate experimental lines in their response to a declining medium concentration were not correlated with differences in their learning score (Pearson's $r = -0.06$, $p > 0.9$ and $r = 0.11$, $p > 0.8$ for orange and pineapple medium respectively).

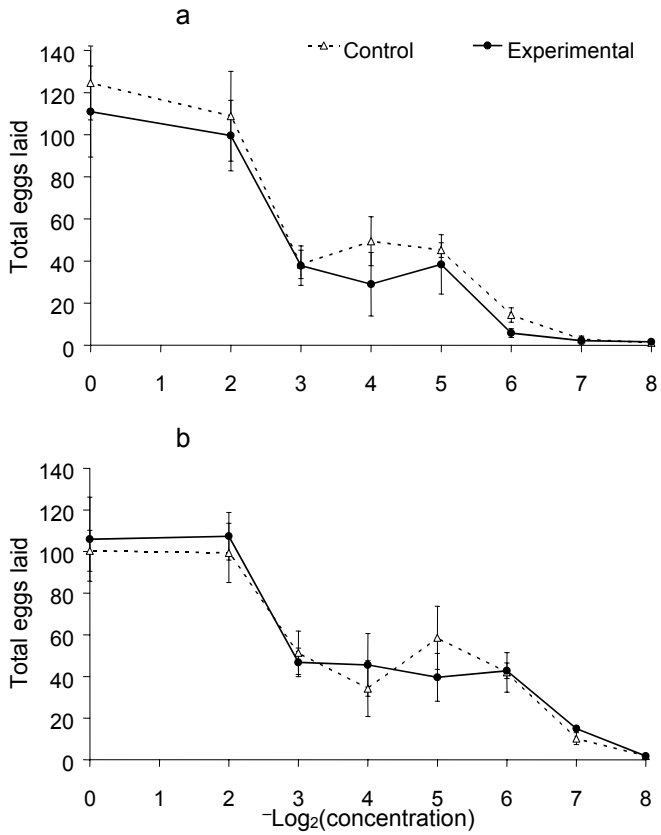

Figure 7. Dose-response curve measuring the potential of diluted fruit medium (a) orange and (b) pineapple) to stimulate oviposition (generation 56). Bars are ± one standard error.

DISCUSSION

When exposed to ecological conditions thought to favor learning, our experimental fly populations evolved an improved ability to associate the taste or smell of an oviposition medium with an aversive chemical cue (quinine) and to avoid this medium several hours later, when the cue was no more present (fig. 4a). We did not observed any significant increase in the number of eggs laid in both treatments over generations. Most of the evolutionary change occurred within the first 20 generations of selection (fig. 2 and 3). The change was partially reversed when the selection regime was suspended and some populations went through a mild bottleneck at generations 27-31. The subsequent slow-down of evolutionary change is likely to reflect loss of genetic variation.

Although the control lines did not show a detectable response to conditioning under the design used during the experimental evolution (fig 4), they did respond to a more intensive and prolonged conditioning regime (fig 5). The experimental flies developed an association between the chemical cue and the medium faster than the control flies, i.e., had a higher learning rate (fig. 5). They also remembered the association longer (fig 6). Hence, both faster learning and better memory contributed to the improved ability of the experimental lines to respond to conditioning. An improved response to conditioning was also observed when the flies were faced with novel fruit media (fig. 4b), i.e., was not limited to the media used during selection.

We can exclude the most plausible alternative explanations. First, even though the experimental lines learned faster than the controls, the conditioned response of both reached the same plateau (Fig. 5). This implies that the effectiveness of quinine as reinforcer (16) does not differ between the two types of lines. (Note that the fact that both lines detect quinine equally well would be insufficient to validate this conclusion (16).) Second, in the absence of conditioning, both types of lines laid the same proportion of eggs on the orange medium, and this proportion was significantly different from 50 % (fig 4a). Similarly, when faced with two novel media – apple and tomato – both experimental and control flies laid about 72% of eggs on the tomato medium in the absence of conditioning (fig 4b). This suggests that the experimental populations did not evolve a substantially better ability to discriminate between the media. However, a firm conclusion on discrimination ability would require a "cross-adaptation" approach (17). Third, the experimental and control lines showed the same oviposition response to decreasing concentration of the media (Fig. 7) and among the experimental lines there was no relationship between this response and the learning ability. Thus, the faster learning response of the experimental flies is not due to greater salience (i.e., perceived intensity) of the odors or tastes of the media as stimuli eliciting oviposition (16).

We cannot yet say how specific the improved learning ability is, i.e., whether it would also be manifested in other learning tasks. The possibility that the improved learning ability of different replicate lines may have different genetic or physiological basis also remains to be addressed. Nonetheless, our study demonstrates that under ecologically relevant circumstances fruit flies can readily evolve improved learning ability and better memory. It supports the theoretical prediction that learning should be favored when the environment is temporally or spatially variable (8) and the animal can get reliable cues (10). It also demonstrates that fruit flies can use their learning ability to modify oviposition substrate choice, with direct consequences for fitness. Other things being equal, under our selection regime the contribution of a fly to the next generation was proportional to the percentage of eggs laid on the favored medium. Based on this assumption, by the 23rd generation the improved learning ability would

already give the flies from the experimental populations a 15 % advantage over the control flies in terms of geometric mean fitness, which is the appropriate measure under a temporally varying selection regime (18).

While using single-locus large-effect mutants is likely to be the most effective approach to uncovering the molecular bases of learning (19), our approach offers an opportunity to study the genetic bases of quantitative variation for learning ability segregating in natural populations (20). It also opens new avenues of research on the ecological consequences and fitness costs of learning.

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CHAPTER 2: A fitness cost of learning ability in *Drosophila melanogaster*

Maintenance of substantial genetic variation for learning ability in many animal populations suggests that learning ability has fitness costs, but there is little empirical evidence for them. In this paper we demonstrate an evolutionary trade-off between learning ability and competitive ability in *D. melanogaster*. We show that the evolution of an improved learning ability in replicated experimental fly populations has been consistently associated with a decline of larval competitive ability, compared to replicated control populations. The competitive ability was not affected by crossing of the replicate populations within each selection regime, excluding differential inbreeding as a potential confounding factor. Our results provide evidence of a constitutive fitness cost of learning ability, i.e, one that is paid irrespective of whether or not the learning ability is actually used.

This paper is in preparation for the Proceedings of the royal society B: biological sciences

INTRODUCTION

Our understanding of how an animal's ability to learn contributes to its Darwinian fitness has recently improved (Johnston 1982; Papaj & Prokopy 1989; Bernays 1998; Dukas 1998; Shettleworth 1999). The most widely accepted idea is that learning allows an individual to adjust its behaviour in an adaptive way in a changing environment, where fitness consequences of a given action vary from generation to generation, or even within a lifetime. Evidence in support of this prediction is growing (Johnston 1982; Papaj & Prokopy 1989; Stephens 1991; Bernays 1998; Dukas 1998; Egas & Sabelis 2001; McNeely & Singer 2001; Mery & Kawecki 2002). In contrast, we know almost nothing about the fitness costs of learning ability (Johnston 1982; Dukas 1999). Yet, learning ability is unlikely to be cost-free. Information processing and storing, as well as development and maintenance of neural structures involved in learning and memory, are energetically costly (Johnston 1982; Bernays 1998; Dukas 1999; Laughlin 2001). As in nature resources are limited, this additional energy expenditure should be reflected in reduction of survival or fecundity: energy and proteins invested in the brain cannot be invested in eggs, somatic growth, or immune system. Hence, learning ability is expected to show evolutionary trade-offs with other fitness-related traits (Dukas 1998, 1999). Maintenance of substantial genetic variation for learning ability in natural populations (McGuire & Hirsch 1977) indicates that learning ability indeed trades off with some fitness components (Rose 1982). Such trade-offs have not been experimentally demonstrated.

To address this problem we tested if evolution of improved learning ability in experimental populations of *D. melanogaster* has been associated with a reduction of another fitness-related trait: larval competitive ability. Measuring such correlated responses to selection is a standard approach used to detect evolutionary trade-offs (Reznick 1985; Stearns 1992). It has recently been successfully used to demonstrate a cost of resistance to parasitoids in *Drosophila* (Kraaijeveld & Godfray 1997). Under natural conditions larval competitive ability is an important fitness trait for fruit flies, which lay many eggs in small patches of an ephemeral resource. We compared the larval competitive ability of two sets of outbred fly lines originating from the same base population. The *High-learning* lines had previously been selected for improved aversion learning with respect to oviposition substrate choice (Mery & Kawecki 2002). The *Low-learning* lines had been maintained under similar conditions, but had not been selected for learning (Mery & Kawecki 2002). To eliminate a potential confounding effect of differential inbreeding, we repeated the assay of larval competitive ability on F_1 crosses between different *High-learning* lines and between different *Low-learning* lines. Both assays indicate that the *High-learning* lines evolved a poorer larval competitive ability as a correlated response to selection for an improved ability to learn.

METHODS

(a) *Fly populations*

We used five *High-learning* and five *Low-learning* lines, originating from a stock established with 2,000 flies collected in Basel (Switzerland) in 1999. The selection regime imposed on the *High-learning* lines favoured flies that could associate the smell of an oviposition substrate with an aversive gustatory cue (quinine hydrochloride), and avoided ovipositing on this substrate several hours later, when the cue was no more present. The details are described elsewhere (Mery & Kawecki 2002). Briefly, every generation flies from the *High-learning* lines were offered a choice between two oviposition media: orange and pineapple. When they first encountered the two media (during a 3-hour *conditioning period*), one of the media (pineapple in odd-, orange in even-numbered generations) was additionally supplemented with quinine hydrochloride. During this conditioning period the flies had an opportunity to associate the smell or taste of the medium with the taste of quinine. During the subsequent 6 hours

(*test period*) the flies were presented with the same two media, both without quinine. The next generation was bred from eggs laid in the second half of the test period (3-6 hours after the end of conditioning) on the medium that had not contained quinine during the conditioning period (i.e., orange in odd-, pineapple in the even-numbered generations). Flies that in the conditioning period learned the association between the medium and quinine, and continued to avoid that medium in the test period, would contribute more genes to the next generation.

The *Low-learning* lines originated from the same base population. They never encountered quinine in the course of selection, and thus were never subject to conditioning. Otherwise, they had been maintained under a similar regime as the *High-learning* lines. That is, they were also offered a choice between the orange and the pineapple medium for oviposition; as in the *High-learning* selection regime, the next generation was bred from eggs laid on the orange medium in odd- and on the pineapple medium in even numbered generations (Mery & Kawecki 2002). In both selection regimes the populations were randomly culled to about 150 adults before oviposition, the generation time was 14 days. Due to an accidental insecticide poisoning in the laboratory at generation 27, the population sizes of most lines in both selection regimes were temporarily reduced (in one case to only about 20 adults). To facilitate recovery, the selection regimes were suspended for generations 27-31, and for other reasons at generations 11 and 35; at those generations the flies oviposited on a standard cornmeal medium.

Within 20 generations of selection the *High-learning* lines evolved a markedly improved ability to respond to conditioning, compared to the *Low-learning* lines (Mery & Kawecki 2002). This improved response to conditioning was due to both faster learning and longer memory, but not due to better discrimination, detection, or salience of (i.e., attention paid to) the stimuli (Mery & Kawecki 2002). The response to selection was not specific to quinine, nor to aversion learning: the *High learning* lines also performed better in a reward learning assay using sucrose as the reinforcer (F. Mery, unpublished data).

(b) *The assay of larval competitive ability*

This assay took place at generation 37 counting from the commencement of selection. Eggs for this assay were collected from each *High-learning* and each *Low-learning* line by allowing 100 mated females (14 days old, counted from egg) to oviposit for 6 h on a grapefruit juice medium. We could not assay the competitive ability of the *High-* and *Low-learning* lines in direct competition with each other due to lack of markers that would allow us to distinguish the lines. Instead, we assayed the competitive ability of each *High-learning* and *Low-learning* line when forced to compete with a standard reference strain, a laboratory strain homozygous for a *white* mutant allele (w^{1118}). This is a standard way of assessing relative competitive ability (Santos et al. 1992; Fellowes et al. 1998). To create competitive conditions we placed 10 eggs from a given line together with 10 eggs of the reference strain in a 5 ml vial containing a pure agar medium with either 125 mg, 50 mg, or 25 mg of dead yeast added. There were four replicate vials per line for each food level treatment. We scored the number of tested (red-eyed) and reference (white-eyed) flies surviving to adulthood.

A competition index was calculated for each line and each food level as the proportion of tested (red-eyed) flies among all surviving flies (pooled over the four replicate vials). The values of the competition index were angularly transformed (Sokal & Rohlf 1995), and analysed with a weighted-least-square analysis of variance, the weight being the total number of surviving flies (WEIGHT statement, PROC GLM of SAS statistical software; SAS Institute Inc. 1989). Unweighted analysis produced qualitatively the same results, so only the weighted analysis is reported.

(c) *Crosses between replicate lines*

Although in both selection regimes the target adult population size was 150 adults per line, fewer adults were available in some lines in some generations (notably at generation 27). Additionally, the variance of the effective reproductive success may have been different between the *High-learning* and *Low-learning* lines, leading to differences in the effective population size. This might have caused some inbreeding, which would have confounded our results, particularly if the degree of inbreeding had been different between the selection regimes, causing differences in larval performance. To see if the lines show evidence of inbreeding depression affecting larval competitive ability, we repeated the competitive ability assay on F_1 crosses between replicate lines within each selection regime. If our lines were indeed suffering from inbreeding depression, these crosses should have at least partially restored heterozygosity and thus show hybrid vigour, except in the highly unlikely case of the same set of deleterious alleles becoming fixed by chance in all populations.

Four F_1 crosses were made at generation 38 by crossing the five experimental lines (line 1 $\times$ line 2, line 2 $\times$ line 3, etc.); four F_1 crosses between pairs of control lines were obtained in the same way. To obtain an F_1 cross between, e.g., line 1 and 2, we mass-mated over 3 days 100 virgin females from line 1 with 100 males from line 2, and 100 females from line 2 with 100 males from line 1. These 200 females were subsequently allowed to oviposit for 6 hours on a grapefruit juice medium; their eggs were pooled. These eggs were used to assay the larval competitive ability of the crosses in the same way as described above for the original lines.

Another sample of 500 eggs from each cross were raised on a standard cornmeal medium at a low density (two bottles/cross, each with 250 eggs on 21ml of medium); the adults developed from them were used to measure the learning ability of the crosses.

(d) *Learning ability of the between-line crosses*

We used the same learning paradigm as that under which the lines had been selected (Mery & Kawecki 2002). From each cross we sampled six groups of 70 adult flies (males + females, 14-days old counting from egg). Each group of flies was transferred to a cage (19 ×12 × 13 cm) and randomly assigned to three treatments (two replicate cages per treatment): (i) conditioned to avoid pineapple, (ii) conditioned to avoid orange and (iii) not conditioned. All treatments involved a choice between an orange and a pineapple oviposition medium. In the treatment "Conditioned to avoid pineapple", naïve flies were first offered one petri dish of each medium, with the pineapple medium supplemented with quinine hydrochloride (4 g/l). This conditioning period lasted 3 hours. Immediately following the conditioning period the flies were offered fresh petri dishes of orange and pineapple medium, neither containing quinine, and allowed to oviposit for 6 hours (test period). Thus, during the conditioning period the flies in this treatment had an opportunity to associate the taste of quinine with the pineapple medium; if they remembered this association they would avoid this medium in the test period. The treatment "Conditioned to avoid orange" was identical except that in the conditioning period quinine had been added to the orange instead of the pineapple medium. In the treatment "Not conditioned" neither medium ever contained quinine. The oviposition site preference of each line was measured as the proportion of eggs laid on the orange medium in the test period, averaged over the two replicate cages. This proportion was analysed separately for the *High-learning* and *Low-learning* lines with a two-way analysis of variance, where the treatment (type of conditioning) was a fixed factor and the cross was a random factor. We also carried out an analysis of variance where the selection regime and treatment were the fixed factors, and the cross was a random factor nested within selection regime. The interaction between selection regime and treatment in this analysis directly tests for the differences in learning ability between the two sets of crosses.

RESULTS

When the quantity of food offered was relatively large, larvae from all lines survived equally well (egg-to-adult survival about 70 %), and so the competitive ability did not differ between the *High-learning* and *Low-learning* lines (weighted-least-square analysis of variance; $F_{1,8}$ = 0.6, p = 0.44; white bars in figure 1a). However, when the quantity of food was more restricted, and thus competition more intense, larvae from the *Low-learning* lines showed higher competitive ability than larvae from the *High-learning* lines ($F_{1,8}$ = 16.5, p = 0.0036, and $F_{1,8}$ = 10.6, p = 0.012, for 50 mg and 25 mg of yeast, respectively; white bars in figure 1b,c). Even under the lowest food quantity the competitive index for the *High-learning* lines did not differ from 0.5, indicating that their competitive ability is not greater than that of the reference strain, which is expected to perform rather poorly due to deleterious pleiotropic effects of the marker allele w^{1118}. These conclusions remained unchanged when we used an alternative measure of competitive ability: $\log_{10}$[(the number of surviving tested flies)/(the number of surviving reference flies + 1)] (Kraaijeveld & Godfray 1997). We also compared the developmental time of the *High-learning* and *Low-learning* flies, but found no difference.

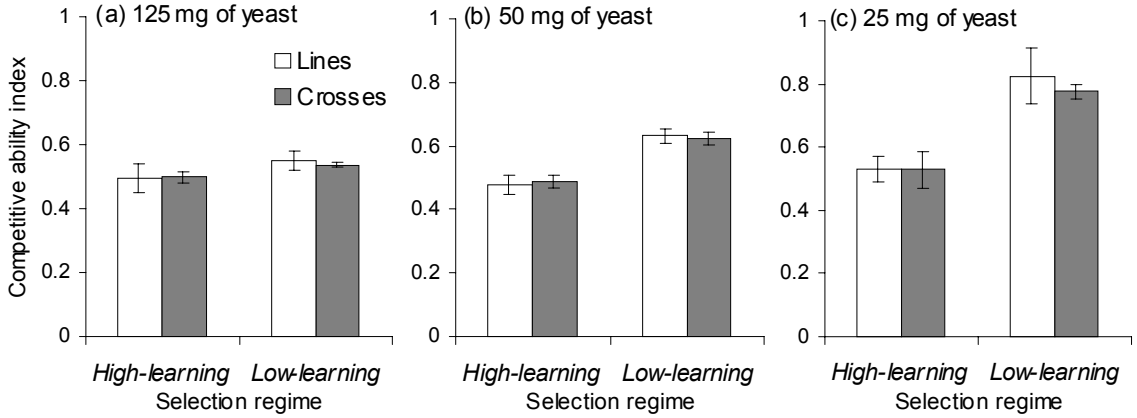

Figure 1: Mean larval competitive ability of the *High-learning* lines (which had evolved an improved learning ability) and *Low-learning* lines (not selected for learning), on three quantities of food. White bars: original selection lines; grey bars: crosses between different replicate lines within selection regimes. Error bars indicate ± one standard error.

The crosses between different replicate *High-learning* lines (measured at generation 38) showed a clear response to conditioning in our learning test (figure 2), although the response was about 40 % smaller than that of the original lines, which, however, were measured at generations 23 and 46 (reported elsewhere; Mery & Kawecki 2002). In contrast, the crosses between different *Low-learning* lines did not respond to conditioning (figure 2). When not conditioned, both types of crosses laid about 58 % of eggs on the orange medium; virtually identical preference has been observed in the original lines and the base population (Mery & Kawecki 2002).

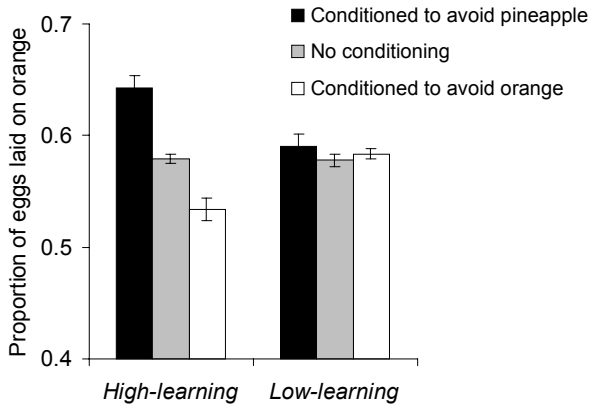

Figure 2: Response of oviposition substrate preference to conditioning, measured for crosses between replicate lines within selection regimes; bars represent ± one standard error. Significant interaction between the selection regime and the conditioning treatment ($F_{2,6}$ = 30.9, p < 0.001) indicates differences in learning ability between the two sets of crosses: crosses between the experimental lines responded to conditioning ($F_{2,6}$ = 30.7, p < 0.001), whereas crosses between the control lines did not ($F_{2,6}$ = 0.9, p = 0.43).

Crossing the lines had no detectable effect on their competitive ability. At all food quantities, and for both *High-learning* and *Low-learning* lines, the average competitive ability of the crosses (figure 1, grey bars) was almost identical to that of the original lines (figure 1, white bars). As was the case for the original lines, crosses between *High-learning* lines had a lower competitive ability than crosses between *Low-learning* lines if food was strongly limiting ($F_{1,6}$ = 22.7, p = 0.003 and $F_{1,6}$ = 17.6, p = 0.005 for 50 mg and 25 mg of yeast, respectively), but not if food was more abundant ($F_{1,6}$ = 4.2, p = 0.08 for 125 mg of yeast). We can thus exclude differential inbreeding as the reason for the differences between the experimental and control lines. Therefore, we interpret the lower competitive ability of the experimental populations as a correlated response to selection for improved learning ability.

DISCUSSION

Animals dependent on learning pay the costs of gaining experience and making mistakes (the costs of being naïve); such costs, involving energy, time and mortality, have been demonstrated in a number of species (Laverty & Plowright 1988; Heinshon 1991; Dukas & Visscher 1994). The cost of learning ability we have demonstrated here is qualitatively different. We believe the correlated response of larval competitive ability to selection on learning ability reflects an evolutionary, genetically-based trade-off (as defined by Stearns 1992). The resulting cost is paid by individuals with genetically high learning ability whether or not they actually make use of this ability. This is, to our knowledge, the first experimental evidence for such a trade-off. Its underlying physiological mechanisms remain to be investigated; it may be mediated by allocation of more resources to the neural and sensory structures underlying learning or memory.

Rather than reflecting pleiotropic effects of genes targeted by selection, a correlated response to selection may be due to genetic hitch-hiking of alleles at loci closely linked to the target genes (Falconer & MacKay 1996). However, the latter mechanism would require that the base population were at considerable linkage disequilibrium. The base population was derived from about 2,000 flies collected at a single locality, and was maintained at the size of several thousand individuals for about 14 generations before selection commenced. It is therefore unlikely for the base population to have been at substantial linkage disequilibrium due to either drift or gene flow. Therefore,

although we cannot exclude a role of linkage, we believe it is more parsimonious to interpret the loss of competitive ability of the *High-learning* lines as a result of pleiotropic effects of alleles improving learning. It is worth noting in this context that many mutant alleles that affect learning in *D. melanogaster* are known to have broad pleiotropic effects (reviewed in Dubnau & Tully 1998).

Based on our results, one would predict that selection for improved larval competitive ability should lead to a loss of learning ability. Similarly, the response to selection for improved learning ability should be slowed down if the populations were simultaneously selected for high larval competitive ability. These predictions remain to be tested. However, evolutionary trade-offs are likely to involve relationships among multiple traits. It would thus not be surprising if simultaneous selection on learning ability and larval competitive ability produced flies with high learning ability and high competitive ability. However, we would then expect that the trade-off should be manifested in reduction of some other fitness component, like longevity, fecundity, or stress resistance. *Drosophila* life history traits are involved in such a flexible trade-off structure, with selection on the same fitness component causing different correlated responses under different conditions and in different populations (Ackermann et al. 2001). We hope that our results will stimulate more studies on evolutionary trade-offs involving learning ability. Ultimately convincing evidence for such trade-offs would come from accumulation of results from many independent studies, using different approaches and different population or species.

Irrespective of the genetic and physiological mechanism of the trade-off, this study demonstrates that to interpret differences in learning ability among populations (Girvan & Braithwaite 1998; Pravosudov & Clayton 2002) or species (Gould-Beierle & Kamil 1998; Lefebvre *et al.* 1997) one needs to compare not only its benefits, but also its costs. It is the balance between the two that will determine whether learning ability will be favoured by natural selection.

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CHAPTER 3: An induced fitness cost of learning in *Drosophila melanogaster*

Abstract: Our understanding of how an animal's ability to learn contributes to its Darwinian fitness has recently improved. The most widely accepted idea is that learning allows an individual to adjust its behaviour in an adaptive way in a changing environment, where fitness consequences of a given action vary from generation to generation, or even within the lifetime. However, the process of collecting and storing information is likely to be costly especially if an individual is subjected within it lifetime to several environmental changes. By exposing during several consecutive days lines of Drosophila melanogaster selected for improved learning ability to repeated conditioning we observed a significant decrease of fecundity and of learning ability compared to the same lines not exposed to such treatment but otherwise kept under similar conditions. The decrease of fecundity was not observed when the treatments were applied to lines not selected for high learning ability indicating that the selected flies paid an induced cost to repeated conditionings. This experiment shows that, if learning can be advantageous when environmental conditions varies between generations, to frequent environmental changes within the life of an individual may counter select the evolution of learning.

this paper is in preparation for animal behaviour

INTRODUCTION

In the recent decades it became clear that many forms of animal behaviour, even in short lived animals, are amenable to learning. The potential ways in which learning could contribute to fitness are diverse (Johnston 1982; Papaj and Prokopy 1989; Dukas 1998). The most widely accepted general idea is that learning allows an individual to adjust its behaviour in an adaptive way in a changing environment, where fitness consequences of a given action vary from generation to generation, or even within the lifetime (Johnston 1982; Papaj and Prokopy 1989; Stephens 1991). Evidence in support these predictions is growing (Simons et al. 1992; Prokopy et al. 1993; Potting et al. 1997; Geervliet et al. 1998; Sol and Lefebvre 2000; Jackson and Carter 2001; McNeely and Singer 2001; Egas and Sabelis 2001; Mery and Kawecki 2002).

While fitness benefits of learning are relatively well studied and understood, we know little about its fitness cost. Understanding the costs is, however, fundamental for understanding why, how, and when learning ability should evolve under natural selection. Several types of costs of learning have been postulated (Johnston 1982, Dukas 1999). The most commonly cited and studied is the cost of being naïve. This includes the cost of errors in trial and error learning, and the time needed before a particular task is mastered. Examples of such cost have been described in foraging bumble bees, which need experience and time before they can handle a new flower properly (Laverty and Plowright 1988), or in bird fledglings which are often unsuccessful in food gathering (Sullivan 1988). Second, an individual with genetically high learning ability may endure some costs whether or not it actually exercises this ability. Such costs can be seen as a pleiotropic effect of genes that improve learning ability. Such costs may be due to development and maintenance of neural and sensory structures involved in learning and memory (Dukas, 1999). For example the size of the hippocampus has been found to be positively correlated with learning ability among several species of birds (Biegler et al. 2001), among genotypes within species (Crusio and Schwegler 1993), and between the sexes (Jacobs and al. 1990). The increase of hippocampus size is likely to be costly in terms of additional structure, which require additional energy (Foley and Lee 1991).

In this study we focused on another type of costs and refer to them as operating costs of learning. Such costs may reflect the energy spent collecting, processing and storing information during the learning process. Little is known about the magnitude of this cost. Neural tissue is known to be metabolically very expensive (Laughlin et al. 1998, Laughlin 2001), although it is not clear to what extent this energy expenditure depends on learning-related activity. Given that energy is a limited resource for animals, such energy expenses are likely to have fitness consequences. If such operating costs exist, an individual which is forced to exercise repeatedly its learning ability should show a reduction in some fitness components, relative to an individual of the same genotype which does not have to learn. Assuming that a higher learning ability is associated with greater operating costs, this difference should be more pronounced in genotypes that show better learning.

To test these prediction, we used laboratory populations of *Drosophila melanogaster* with different selection history. The “High learning” lines had been selected for high learning ability and improved memory capacities in the context of oviposition substrate choice (Mery and Kawecki, 2002). The “Low learning” lines, maintained under the same conditions, had not been exposed to this selection and only show poor learning ability and memory. From each “High learning” and “Low learning” line we derived two samples of freshly emerged flies. One sample was exposed everyday to a learning task (the *Conditioned* treatment) whereas the other sample was kept under similar conditions but without learning task (the *Not Conditioned* treatment). If learning imposes a fitness cost, we expected that “High learning” flies of the *Conditioned* treatment would show a decrease of their fitness (survival or fertility) compared to flies from the same “High learning” lines kept under the *Not Conditioned* treatment. Following

the same hypothesis, we expected that the exposure to repeat conditionings will have little effect on the “Low learning” flies.

MATERIAL AND METHODS

Selection lines: We used five “High learning” and five “Low learning” lines. All these lines originated from the same base population, collected in Basel (Switzerland) in 1999. The “High learning” lines had been selected for improved aversion learning with respect to oviposition substrate choice. The selection regime favoured flies that could associate the smell of an oviposition substrate (orange or pineapple) with an aversive gustatory cue (quinine), and avoided ovipositing on this substrate several hours later, when quinine was no more present. The “Low learning” lines had not been exposed to selection for learning, but otherwise maintained under the same conditions. The details are described elsewhere (Mery and Kawecki, 2002). Within 20 generations of selection the “High learning” lines had evolved a markedly improved ability to respond to conditioning, compared to the “Low learning” lines. The improvement was due to both faster learning and longer memory of the “High learning” flies, but not due to better discrimination, detection, or salience of (i.e., attention paid to) the stimuli (Mery and Kawecki, 2002). The assay reported here was done after 57 generations of selection.

Design of the experiment: The assay was carried out in cages (19 × 12 × 13 cm). At the bottom of each cage two petri dishes (diameter 60 mm) with oviposition media were attached; they could be exchanged with little disturbace to the flies. We used an orange and a pineapple medium, prepared from 100% orange or pineapple juice from concentrate, with 6.6 g/l agar, and a drop of live yeast added on top; these are the two media that had been used in the course of selection. When the flies needed to be conditioned (see below), one of the media was supplemented with quinine (4 g/l of quinine hydrochloride), which is a repulsive gustatory reinforcer. Each time two different media were being offered, their position was randomized. We also used a non-nutritional, pure agar medium, with two drops of live yeast on top. Throughout the assay, except for the brief periods when the media were being exchanged, the cages were maintained in complete darkness, at 25°C and 70 % relative humidity.

From each “High learning” and “Low learning” line we derived two samples of 50 males + 50 females. Each sample was placed in a separate cage. All flies were freshly emerged (aged 14 days counted from egg). One cage was assigned to treatment *Conditioned*, the other to treatment *Not conditioned*.

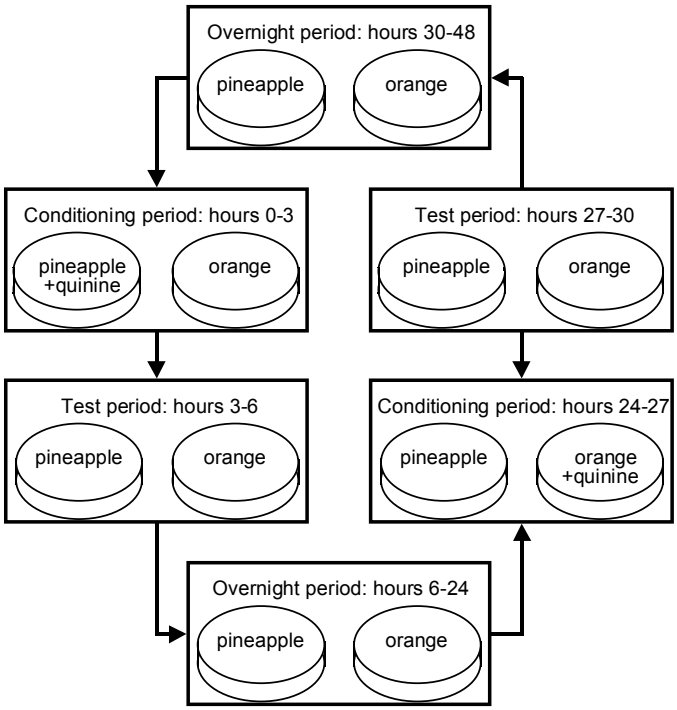

Figure 1: Design of a conditioning cycle for the *Conditioned* treatment. Each cycle is divided in two days of conditionings (Conditioning to avoid pineapple the first day and then conditioning to avoid orange the second day). Between these two days, flies were kept in the cage and were offered media containing pure agar and a drop of yeast. In the Not Conditioned treatment flies were kept exactly the same way except that no conditioning occurred.

In treatment *Conditioned* the flies were subjected to repeated 48-hour cycles of conditioning (Fig 1). During the first 3 hours of each cycle (first conditioning period, hours 0-3) the flies were conditioned to avoid pineapple: they were offered both fruit media, whereby the pineapple medium was supplemented with quinine. During the following 3-hour period (first test period, hours 3-6) the flies were offered a fresh set of the orange and pineapple medium, this time neither containing quinine. Based on previous assays (Mery and Kawecki 2002) we expected that the "High learning" flies would remember the association between the pineapple medium and quinine and continue to avoid ovipositing on it within the test period, in spite of quinine not being present any more. In contrast, "Low learning" flies had never showed this ability (Mery and Kawecki 2002). For the following 18 hours (overnight) the flies were only offered the pure agar medium. This way daily oviposition was concentrated within six hours (very few eggs were laid on the pure agar medium). Additionally, this likely caused some nutrient-limitation and stress for the ovipositing flies; life history tradeoffs are more likely to be detected under stressful conditions (Leroi et al 1994). On the second day of each cycle the flies were conditioned for three hours to avoid orange (second conditioning period, hours 24-27), followed by three hours of oviposition on orange and pineapple medium not containing quinine (second test period, hours 27-30), and 18 hours (hours 30-48) on pure agar (Fig. 1).

In treatment *Not Conditioned* the flies were treated exactly in the same way except that they were never given any medium containing quinine. In both treatments we counted the number of eggs laid in every cycle during both test periods (hours 3-6 and 27-30) on the orange and on the pineapple medium. The half of total number of eggs laid over a cycle gives an estimate of the daily productivity of the flies. As some flies died during the experiment, differences in productivity may reflect differences in fecundity as well as in the number of surviving flies; because we did not monitor the cages for dead flies, we cannot separate these two components of productivity. After 12 consecutive cycles (i.e. 24 days) we terminated the experiment and counted the number of surviving flies.

Control for the effect of ageing: *Resting* treatment. Flies usually show a loss of both fecundity and learning ability as they age, even under standard laboratory conditions. This ageing effect might be a potential confounding factor in our experiment. To control for this effect, we kept in parallel to the above treatments three samples of 50 males + 50 females from each "High learning" line. These flies had the same age as those used in the assay described above. The first of these three samples was maintained for 7 days on a standard cornmeal medium, and then subject to a single 48-hour conditioning cycle identical as in treatment *Conditioned* (Fig. 1). The second and third sample were similarly subjected to a single conditioning cycle after 14 and 21 days, respectively, on the standard medium. We refer to this treatment as *Resting*. We could therefore compare the productivity and learning ability of the "High learning" flies subject to the *Conditioned* treatment (which were forced to learn daily) to flies of the same lines and same age maintained under standard conditions. This would permit us to separate the effects of repeated exposure to conditioning from the effects of ageing.

Statistical analysis: In this experiment we therefore have 2 treatments (*Not Conditioned* or *Conditioned*) applied each to 2 selection regimes ("Low learning" or "High learning") composed of 5 lines each.
SAS v8.02 statistical package was used for all analysis. In the *Conditioned* treatment, quinine was added to one of the media offered during the first period. The distribution of eggs laid in this period therefore simply reflected avoidance of quinine. This avoidance was always almost complete for the "Low learning" and the "High learning" lines (less than 2 % of eggs were laid on the quinine-containing medium), and is of no interest here. Therefore, only eggs laid during the second period were included in all following analysis.

For the *Conditioned* treatment, we calculated a learning score for each replicate line and each cycle. The learning score was calculated as the difference between the proportion of eggs laid on orange when flies where conditioned to avoid pineapple (i.e. on the first day of each cycle) and when flies where conditioned to avoid orange (i.e. on the second day of each cycle). Each proportion was previously arcsin ($x^{1/2}$) transformed before analysis.

To analyse the effect of repeated rounds of conditioning on the learning score of the "High learning" and "Low learning" *Conditioned* flies, we performed an ANCOVA using cycle as covariates, and selection regime as a fixed factor and line (nested within selection regime) as random factor. All main effects and interactions were included in the model.

Productivity was measured as the total number of eggs laid during the second period averaged over the two days of each cycle. We analysed the changes of productivity (log transformed) in the course of the experiment using an ANCOVA with cycle as a covariate, selection regime ("High learning" or "Low learning") and treatment (*Conditioned* or *Not Conditioned*) as fixed factors and line (nested within selection regime) as random factor. All main effects and interactions were included in the model.

We then analysed the effect of repeated rounds of conditioning on the realised productivity for "High learning" and "Low learning" flies. For each replicate line and cycle, we calculated the ratio of productivity between the *Conditioned* treatment and the *Not Conditioned* treatment. This ratio was log transformed before the analyses. We then performed a repeated measure ANCOVA using cycle as a covariate, selection regime ("High learning" or Control) as a fixed factor and line (nested within selection regime) as a random factor.

For each selection regime, we analysed the relationship between productivity and learning score among *Conditioned* replicate lines throughout the experiment. This was done with a multiple regression analyses on the productivity ratio (log transformed) with cycle and learning scores (calculated from arcsin ($x^{1/2}$) transformed

proportions) as covariates. A significant effect of the interaction cycle × learning score would indicate that the slope of the relationship between productivity and learning score among replicate lines changed in the course of the experiment.

In all ANCOVA including cycle as a covariate we also tested non-linearity by first including in the model cycle^2 as a covariate. If not significant, this effect was removed from the model.

RESULTS

Learning score. The learning score of flies subject to the *Conditioned* treatment was consistently higher for the "High-learning" than for the "Low-learning" lines (Fig. 2, Table 1), and changed over time in a non-linear way. The learning score of the “High learning” lines initially increased, reaching a maximum aroung the 6th cycle, and then decreased (Table 2), so that it was significantly smaller in cycles 10-12 than in cycles 1-3 ($F_{1,20}$ = 8.69, P = 0.008). When analysed separately, the decrease of learning ability between the 3 last cycles compared to the 3 first was observed for the “High learning” lines 2 ($F_{1,4}$ = 7.76, P = 0.04) and 5 ($F_{1,4}$ = 11.01, P = 0.02) but was not observed for the “High learning” lines 1, 3 and 4 (all P > 0.3).

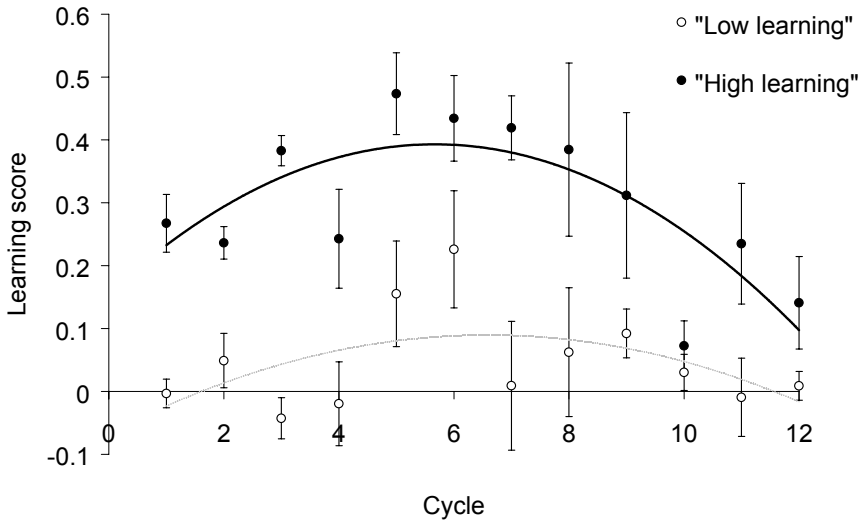

Figure 2: The effect of repeated rounds of conditioning on the learning score of “High learning” and “Low learning” flies. For each replicate line and cycle, the learning score was calculated as the difference between the proportion of eggs laid on orange when flies were conditioned to avoid pineapple and when flies were conditioned to avoid orange. These proportions were previously arcsin ($x^{1/2}$) transformed. The trend lines are quadratic regressions.

Table 1: Results of the ANOVA on the learning score. The numbers in the "denominator" column refer to the effect used as error term. All non significant interactions ($P > 0.05$) were removed from the model.

Effect	denominator	F value and P value
1- Line(selection regime)	5	$F_{8, 107} = 1.1,\ P = 0.33$
2- Selection regime	1	$F_{1, 8} = 59.4, P < 10^{-3}$
3- Cycle	5	$F_{1, 107} = 11.7, P < 10^{-3}$
4- Cycle^2	5	$F_{1, 107} = 14.6,\ P < 10^{-3}$
5- residual		

In contrast to the changes of learning score in the *Conditioned* treatment, the learning score of the "High learning" kept under standard conditions until the test (treatment *Resting*) did not change with time ($F_{2,8}$ = 0.48, P = 0.77). Their learning score measured after 14 days tended to be lower than that of the same lines under the *Conditioned* treatment, the reverse was the case after 22 days (Fig. 3) (ANOVA: test × treatment: $F_{1,8}$ = 6.3 P = 0.03).

Table 2: Results of the ANOVA on the learning score. The analysis was done for each selection regime separately. The numbers in the "denominator" column refer to the effect used as error term. All non significant interactions ($P > 0.05$) were removed from the model.

Effect	*denominator*	*High learning*	*Low learning*
1- Line	4	$F_{4, 53} = 1.1,\ P = 0.35$	$F_{4, 52} = 1.3,\ P = 0.28$
2- Cycle	4	$F_{1, 53} = 8.2,\ P = 0.0061$	$F_{1, 52} = 3.9,\ P = 0.055$
3- Cycle^2	4	$F_{1, 53} = 11.4,\ P = 0.0014$	$F_{1, 52} = 3.9,\ P = 0.06$
4- residual			

The learning score of the "low learning" also showed a similar tendency of first increasing and then decreasing (Table 2) however it never differed from zero (t test, all $P > 0.1$).

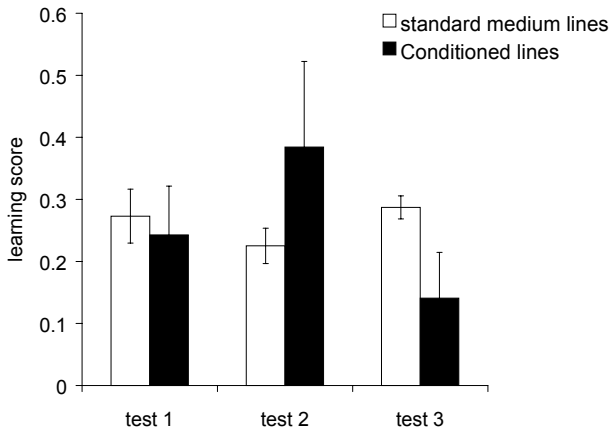

Figure 3: Comparison of the learning score (± standard errors) of the “High learning” Conditioned lines and the same lines but kept without conditioning on a standard food medium (Resting treatment). Test 1 was done after 3 cycles of repeated conditioning, Test 2 was done after 6 cycles and Test 3 was done after 9 cycles.

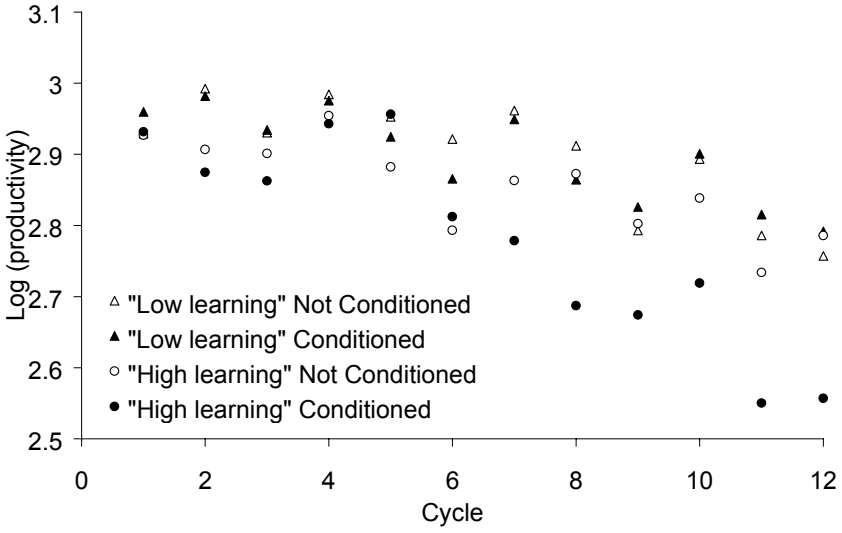

Figure 4: Evolution of the averaged productivity (log transformed) over cycles for the “High learning” and “Low learning” selection regime lines and for the *Conditioned* and *Not Conditioned* treatments.

Table 3: Results of the ANOVA on the productivity of flies (log transformed) including all treatments and selection regimes. The numbers in the "denominator" column refer to the effect used as error term.

Effect	denominator	*F* value and *P* value
1- Line(selection regime)	9	$F_{8,8} = 0.5$, $P = 0.81$
2- Cycle	12	$F_{1,199} = 46.6$, $P < 10^{-3}$
3- Selection regime	1	$F_{1,8} = 0.01$, $P = 0.93$
4- Treatment	9	$F_{1,8} = 0.2$, $P = 0.66$
5- Cycle × line(Selection regime)	12	$F_{8,199} = 1.5$, $P = 0.15$
6- Cycle × Selection regime	12	$F_{1,199} = 3$, $P = 0.0081$
7- Cycle × Treatment	12	$F_{1,199} = 3.1$, $P = 0.071$
8- Treatment × Selection regime	9	$F_{1,8} = 0.1$, $P = 0.72$
9- Treatment × line(selection regime)	12	$F_{8,199} = 1.7$, $P = 0.09$
10- Cycle × treatment × line(selection regime)	12	$F_{8,199} = 3.05$, $P = 0.002$
11- Cycle × Selection regime × Treatment	12	$F_{1,199} = 3.5$, $P = 0.03$
12- Residuals		

Fly productivity. At the beginning of the experiment, the productivity of the flies did not differ between the selection regimes and the treatments (Fig. 4, Table 3). The productivity of the flies decreased with time in all treatments and selection regimes (Fig. 4). However, the rate of decrease was different between selection regimes and treatments (Table 3). The reasons for the three-way interaction among cycle, treatment and selection regime are revealed by the separate analysis of the two selection regime (Table 4). We detected no difference in the rate of decrease productivity between "High learning" and "Low learning" lines when not exposed to repeated conditioning but a difference when exposed to conditioning (Table 4). The analysis based on the productivity ratio indicated that the effect of repeated rounds of conditioning on the realised productivity of the flies differed between "High learning" and "Low learning" lines (Fig. 5, Table 5). For the "High learning" lines, this ratio declined with time and the rate of decline was different among lines (line: $F_{4,50} = 1.6$, $P = 0.2$; cycle: $F_{1,50} = 16.1$, $P = 0.0002$; cycle × line: $F_{4,50} = 6.2$, $P = 0.0004$): "High learning" lines 1 and 3 showed a strong productivity ratio decrease ($F_{1,10} = 9.8$, $P = 0.01$ and $F_{1,10} = 15.1$, $P = 0.003$ respectively) whereas "High learning" lines 2, 4 and 5 did not showed such a decrease (all $P > 0.2$). For the "Low learning" lines we did not observed any decrease of the productivity ratio over time (line: $F_{1,49} = 1.0$, $P = 0.4$; cycle: $F_{1,49} = 0.6$, $P = 0.42$) and this was true for all "Low learning" lines (all $P > 0.2$).

Table 4: Results of the ANOVA on the productivity of the flies (log transformed). the analyses was done separately for the *Conditioned* and the *Not Conditioned* treatment. The numbers in the "denominator" column refer to the effect used as error term.

Effect	*denominator*	Conditioned	Not Conditioned
1- Line(selection regime)	6	$F_{8,99} = 1.3$, $P = 0.25$	$F_{8,100} = 1.2$, $P = 0.28$
2- Cycle	6	$F_{1,99} = 24.6$, $P < 10^{-3}$	$F_{1,100} = 25.3$, $P < 10^{-3}$
3- Selection regime	1	$F_{1,8} = 0.08$, $P = 0.78$	$F_{1,8} = 0.1$, $P = 0.73$
4- Cycle × line (selection regime)	6	$F_{1,99} = 2.8$, $P = 0.007$	$F_{1,100} = 0.7$, $P = 0.67$
5- Cycle × selection regime	6	$F_{1,99} = 4.3$, $P = 0.03$	$F_{1,100} = 0.02$, $P = 0.87$
6- Residuals			

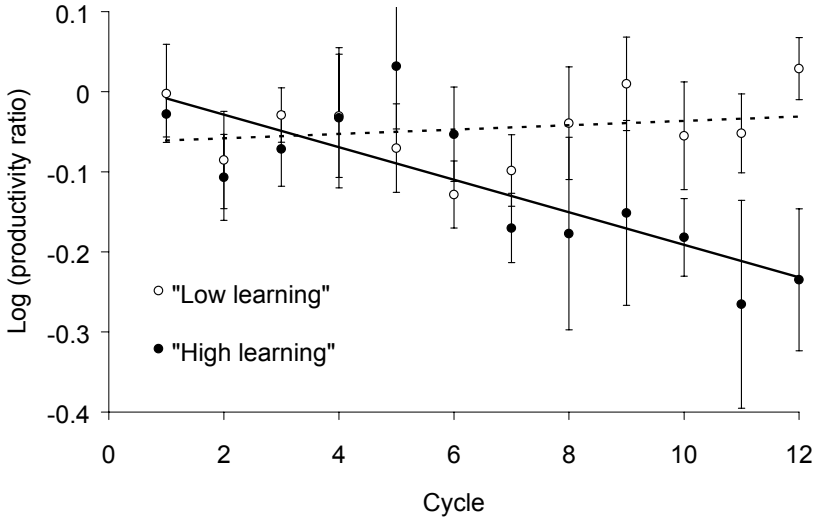

Figure 5: The effect of repeated rounds of conditioning on the realised productivity of flies from the "High learning" and "Low learning" lines. For each replicate line and cycle we divided the averaged number of eggs laid by flies exposed to a daily conditioning routine by the averaged number of eggs laid by flies from the same line not exposed to conditioning. The graphs show means of this ratio (log transformed) across lines as a function of the conditioning cycle since the beginning of the assay.

Table 5: Results of the ANOVA on the productivity ratio (log transformed). The numbers in the "denominator" column refer to the effect used as error term.

Effect	*denominator*	F *value and* P *value*
1- line(selection regime)	6	$F_{8, 99} = 1.4$, $P = 0.21$
2- Cycle	6	$F_{1, 99} = 6.6$, $P = 0.011$
3- Selection regime	1	$F_{1, 8} = 1.9$, $P = 0.2$
4- Cycle × selection regime	6	$F_{1, 99} = 12.9$, $P < 10^{-3}$
5- Cycle× line(selection regime)	6	$F_{8, 99} = 4.2$, $P < 10^{-3}$
6- residual		

Correlation between productivity and learning score. For the "Low learning" lines, there was no relationship between productivity ratio and learning score and this did not change with repeated conditioning cycles (Table 6). On the other hand, for the "High learning" selection regime, we observed a negative relationship between productivity and learning score. This relationship increased with repeated conditioning cycles (Table 6). This is illustrated in Figure 6, which shows that for the first conditioning cycles no correlation was found between productivity ratio and learning score (Fig. 6 a, b) but for the last conditioning cycles (Fig. 6 c, d) we observed a significant negative correlation between productivity ratio and learning score. The results were unchanged when productivity of the conditioned treatment was used instead of productivity ratio.

Survival comparison: After the 24 days of the experiment 70% (±8.5) of the initial flies were still alive in each cage. We did not observe any difference between selection regimes ($F_{1,16}$= 0.01, P = 0.93), nor between treatments ($F_{1,16}$ = 0.01, P = 0.94), indicating that the exposure to quinine had no effect on fly survival. We also did not observe any difference of survival between male and female flies (Paired t test: t = -0.72, P = 0.47). This indicates that the data concerning productivity were roughly based on similar fly numbers in all treatments and selection regime.

Table 6: Results of the multiple regression analyses done on fly productivity ratio(log transformed). The analyses was done for the *Conditioned* treatment each selection regime separately.

	High Learning			*Low learning*		
	Partial regression coefficient	*F value*	P value	*Partial regression coefficient*	*F value*	P value
Cycle	*0.004*	$F_{1, 56}$ = 0.1	P = 0.68	*0.003*	$F_{1, 55}$ = 0.6	P = 0.4
learning score	*0.607*	$F_{1, 56}$ = 3.1	P = 0.08	*-0.49*	$F_{1, 55}$ = 2.7	P = 0.1
Learning score× Cycle	*-0.11*	$F_{1, 56}$ = 7.7	P = 0.007	*0.02*	$F_{1, 55}$ = 0.4	P = 0.5

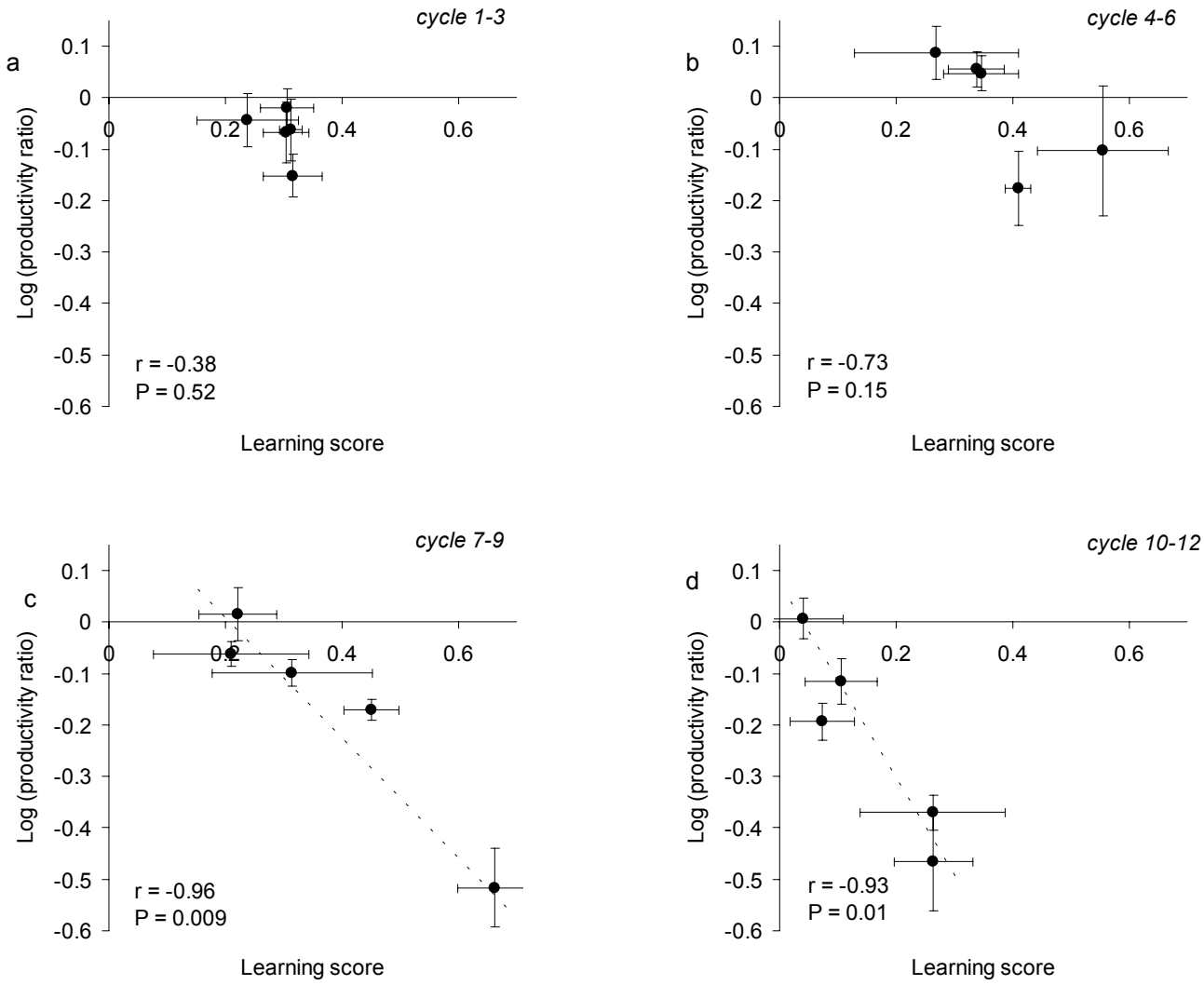

Figure 6: The effect of repeated rounds of conditioning on the correlation between learning score and productivity ratio (log transformed) for the “High learning” *Conditioned* lines. Each graph was made by pulling the data over three consecutive cycles of conditioning for each replicate line. The lines are least-square regression calculated from the averaged productivity and learning score per replicate line. Results of Pearson correlation test are given.

DISCUSSION

The aim of this study was to address experimentally the fitness cost induced by the learning process. We expected that flies exposed to repeated cycles of conditioning would show a decline in productivity or survival, relative to flies of the same lines kept under similar conditions but not exposed to repeated conditioning. Furthermore, if this decline should reflect a cost of learning, we expected that it would be more pronounced in the "High-learning" lines than in the "Low-learning" lines, because the former showed a better learning ability. We also expected that being exposed day after day to alternating conditioning will lead to a progressive decline of the learning score.

As predicted based on previous work (Mery and Kawecki, 2002), the “Low learning” flies did not detectably modify their oviposition preference in response to conditioning. In contrast, the “High learning” flies showed significant learning ability: they were able to associate a medium with an aversive cue (quinine) and continued to avoid this medium after the cue has been removed (Fig. 2). They were responding to conditioning day after day,

even though the direction of conditioning was altered between consecutive days. In contrast to our prediction, their learning score tended to increase during the first part of the experiment, reaching a maximum around the 6th cycle (i.e., the 12th day of the experiment). This may reflect acclimation of the flies to the task they were facing, which consisted of associating always the same cue to one of two fruit media. However, after the 6th cycle of conditioning the learning score of the flies began to decline. This decline was not observed in flies from the same lines and of the same age when maintained until the day of the assay under standard conditions, without being subject to cycles of conditioning (Fig. 3). Thus, although flies are known to lose their learning ability at old age (Fresquet and Medioni, 1993), simple ageing cannot explain the decline in learning ability of flies subject to repeated cycles of conditioning.

At the beginning of the experiment, the productivity of the flies was similar for all treatments and selection regimes and decrease progressively in the course of the experiment. We could not detect any difference of productivity between “High learning” and “Low learning” lines when not exposed to conditioning. This indicates that selection for improved learning ability in the "High-learning" lines did not lead to a constitutive reduction of fecundity as a correlated response. However, while repeated conditioning did not affect the productivity of the “Low learning” lines, flies from the “High learning” lines subjected to repeated rounds of conditioning showed a progressive reduction of productivity relative to the same lines not subject to conditioning.

Furthermore, exposure to repeated rounds of conditioning revealed an apparent trade-off between the learning score and productivity among the “High learning” lines. The effects of repeated cycles of conditioning varied somewhat among different “High learning” lines. Some of them maintained a relatively high learning ability until the end of the experiment, but showed a greater reduction of productivity in the *Conditioned* than in the *Not Conditioned* treatment. In other "High-learning" lines the learning score towards the end of the experiment was low, but their productivity remained relatively unaffected by the treatment. Thus a negative correlation developed in the course of the experiment between the learning score and productivity (Fig. 6).

We interpret the general decrease of productivity or of learning ability as a response of an accumulation of stress due to the experimental conditions. During the 24 days of experiment the flies were kept under conditions which might be partially stressful even for the *Not Conditioned* treatment: the amount of food was limited (only fruit medium 6h a day and a small amount of yeast overnight were offered), the opportunities for oviposition were limited (“good” oviposition substrates were only offered 6h a day) and the flies were often disturbed. Such stressful conditions and ageing might explain the general reduction of productivity observed in all lines especially when compared to the productivity of flies of the same age but kept always on standard food medium. However, the daily addition of quinine to one medium might have induced a differential stress for “High learning” than “Low learning” flies. We propose three alternative hypotheses to explain this effect.

“High learning” flies might have evolved higher susceptibility to quinine. Even if “High learning” and “Low learning” lines showed the same complete aversion to quinine, its toxic effect may be greater in the “High learning” flies. According to the stimulus-response reinforcement principle (Thorndike, 1911), this might be an explanation for the better learning score observed in this selection regime line: a strong punishment produces stronger association than a weak one, and strong association produces better performance than weak association. By evolving a higher susceptibility to quinine, “High learning” flies would therefore show higher learning abilities than “Low learning” flies but would suffer more from the toxicity of the chemical. Consequently, one would expect a stronger reduction of productivity due to the accumulation of additional stress. However, in previous conditioning tests (Mery and Kawecki, 2002) we could not detect any difference of salience of quinine between the two selection regimes and,

even if small differences could have been cumulated in the course of the present experiment, it seems unlikely that they would induce such a strong effect on productivity.

Another hypothesis is the induction of frequent environmental changes in our “High learning” design. The daily change of the position of quinine in one or the other fruit media might have been too rapid to be tracked by the flies. The process of information storing requires protein formation, creation of new neuronal connections and the degradation of outdated information. A fly which has to learn repeatedly one day to reject one medium and the other day to accept it may not be able to “update” completely the new information, traces of old information may accumulate and interfere with the new one. This process known as interference has been demonstrated in insects like bumble bees (Gegear and Laverty, 1998) and has been proposed to be one of the major constraints on animal learning in nature. Such accumulation of outdated information would be reflected by a decrease of the observed learning score of the flies and may also induce a reduction of productivity. The more flies are alternatively conditioned to avoid one medium and then the other one, the more both media might appear toxic for a fly which will avoid laying eggs on any of the two. In this sense, the interference process can be seen as a cost induced by learning. However, previous conditioning tests have shown that “High learning” flies loose a large part of their memory for oviposition site within a day (Mery and Kawecki, 2002) and interference should therefore only play a minor role. Moreover, if interference would induce a reduced learning score and a reduced productivity we would expect the establishment of a positive correlation between learning score and productivity which is in contradiction with the present results.

A last explanation is that the process of learning and memory might have itself induced additional costs. The storage of new information and the destruction of outdated ones may require energy. The more flies had to learn, the higher may become the demand of energy. This additional energy expenditure may be reflected by a reduction of productivity as energy and proteins invested in the brain cannot be invested into egg production. Following this scenario we expect a negative correlation between learning score and productivity. The reduction of learning ability observed for some “High learning” lines but not for others might reflects the evolution of different strategies to face potential stress conditions among replicates lines which have now evolved independently for almost sixty generations. If we could not detect any differences of learning abilities or productivity among these lines when kept under standard conditions (Mery and Kawecki 2002) the allocation of resource seems to have evolved differently. When faced to limited resource and conditioning, some lines allocate more to the process of learning at the expense of productivity whereas some lines do the reverse.

Independently of the process by which “High learning” lines faced additional stress in the *Conditioned* treatment, the negative relationship between productivity and learning observed in the last cycles (Fig 5 c, d) indicate that “High learning” flies may have evolve different strategies to face a potential stress and that exercising learning might be costly. This is, to our knowledge, the first experimental demonstration of such a trade off, more experiment are needed to confirm this effect and its underlying physiological mechanisms remain to be investigated. It shows that the process of learning itself might induce cost when it is extensively used.

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CHAPTER 4: Evolutionary interactions between the innate and learned components of behavior: experimental evolution of oviposition substrate preference in *Drosophila melanogaster*

Learning is thought to be adaptive in variable environments, while constant, predictable environments are supposed to favor unconditional, genetically fixed responses. A dichotomous view of behavior as either learned or innate ignores a potential evolutionary interaction between the learned and innate components of a behavioral response. We addressed this interaction in the context of oviposition substrate choice in *Drosophila melanogaster*, asking two main questions. First, can learning also evolve in a constant environment that favors a fixed preference? Second, how does an opportunity to learn affect the evolution of the innate component of oviposition substrate choice? We exposed experimental populations to four selection regimes involving selection on oviposition substrate preference (an orange versus a pineapple medium). In two selection regimes flies were selected for innate preference either for the orange medium, or for the pineapple medium. In the remaining two selection regimes the flies were also selected for preference for either orange or pineapple, but additionally could use aversion learning to decide which medium it paid to avoid. Lines exposed to the latter selection regimes evolved improved learning ability, indicating that learning may be advantageous even if the same behavioral response is favored every generation. Furthermore, of the two selection regimes that favored oviposition on the pineapple medium, the regime that allowed for learning led to the evolution of a stronger innate preference for pineapple than the regime that did not allow for learning. We propose a simple graphical model that could provide a theoretical framework explaining this synergetic effect between learning and the evolution of the innate component of oviposition substrate preference.

This paper is in preparation for Evolution

Behavioral responses of an animal depend on an interaction between an innate and a learned component. The innate, heritable component reflects the evolutionary history of the population. The learned component is affected by experience accumulated within the individual's lifetime. The ability to modify behavior based on experience (i.e., learning ability) is itself a product of evolution, with notable genetic differences between related species or even conspecific populations (Gould-Beierle and Kamil 1998; Girvan and Braithwaite 1998; Jackson and Carter 2001). It has been predicted that learning would be advantageous in variable environments, i.e., when the fitness consequences of a given behavioral action change from generation to generation, or even within an individual's lifespan (for reviews see Johnston 1982; Stephens 1991; Dukas 1998). (Obviously, for learning to be useful the environment must not change too fast relative to how fast the animal can learn; Dukas 1998.) On the other hand, gaining experience is often costly and error-prone (Laverty and Plowright 1988; Sullivan 1988), and the energy spent on information processing and the maintenance of underlying structures may also entail fitness costs (for reviews see Johnston 1982; Mayley 1996). Hence, innate, ready to use behavioral responses are expected to be favored in constant environments, in which the same behavioral response is always optimal (Dukas 1998).

A dichotomous view of behavior as either mostly learned or mostly innate ignores the importance of the interaction between the evolution of the innate component and the evolution of learning ability. This interaction is the focus of this paper. We pose two questions. First, will learning ability also be selected in a stable environment, where the same behavioral response is always favored? Second, does learning ability affect the evolution of the innate component? We addressed these questions, concentrating on oviposition substrate choice (or more generally diet choice) as the focal behavior, and using experimentally evolving *Drosophila melanogaster* populations as the model system.

The above two questions have been addressed with verbal arguments, and mathematical and computer models. Papaj (1994) studied a model in which genetic variation existed for both the innate component of the response and for learning ability, and the animal could get a feedback from the environment about the fitness consequences of its previous behavioral actions (i.e., reinforcement). Results of that model suggest that directional selection on a behavioral response may lead to the evolution of improved learning ability. Models addressing the second question led to contradictory predictions. On the one hand, learning may allow an individual to behave optimally irrespective of its genotypic value for the innate component of the behavior. Thus, the ability to learn partially decouples the behavioral phenotype from the genotype. It has been proposed that this effect should make natural selection on the innate, heritable component of the behavioral response less effective, slowing down its evolution (Papaj 1994; Robinson and Dukas 1999). In contrast, a simulation model by Hinton and Nowlan (1987) suggests that in a novel environment learning may accelerate the evolution of the innate component towards the optimum. A similar prediction has been obtained in artificial intelligence models (Belew 1989; Ackley and Littman 1991; French and Messinger 1994; Mayley 1997). These models provide some formal underpinning for the old verbal arguments that learning may accelerate evolution (Baldwin 1896; Morgan 1896; Osborn 1896). However, quantitative genetic models making more general assumptions about the fitness function (Anderson 1995; Ancel 2000) suggest that the conditions under which learning may facilitate evolution are highly restricted. Whether learning accelerates evolution should depend on the costs of learning and on the relationships between the genotype and the phenotype.

Rather than attempting a detailed analysis of those models, we illustrate the basic argument with a simple graphical model. The model is particularly suitable in the context of oviposition substrate or diet choice. Consider an animal faced with a choice between two resource types, which it can distinguish (e.g., two types of fruit for a fruit

fly). Each individual has an innate, genetically determined preference ranging from 0 (absolute preference for resource A), through 0.5 (no preference), to 1 (absolute preference for resource B). In the absence of learning, this innate preference directly translates into the mean behavioral response (e.g., the proportion of eggs laid on each fruit type). Now, assume that resource B is of low quality and the animal can perceive this after inspection (e.g., the fruit has low nutritional value or contains toxic secondary compounds). If the animal is capable of learning, it should modify its behavioral response towards greater avoidance of resource B. The effect of learning on the behavioral response will depend on how fast the animal learns, and will increase with the number of experience instances (e.g., oviposition attempts); we assume that both are limited. However, the extent to which learning modifies an individual's realized preference is also likely to depend on its innate preference, especially if the learning rate and the number of experiences are limited. If an individual already has a strong innate aversion of resource B (i.e., strong preference for resource A), the effect of learning on the behavioral response will be small simply because there is little scope for a further increase of aversion. It is less obvious how strong the effect of learning will be in individuals with strong preference for resource B. An individual with an absolute innate preference for resource B will never visit A, so it will have no opportunity to learn that A is better. If the preference for B is somewhat less extreme but still strong, the individual will occasionally experience A and find that it is better, i.e., its experience will conflict with the innate expectation. Evidence from phytophagous insects (e.g., Potter and Held 1999) and humans (Ohman and Dimberg 1978) suggests that in this case the effect of learning on the realized preference is also likely to be relatively small; many repeated instances of negative experience will be needed to shift the individual's preference away from its initially favorite resource. Thus, based on the above arguments, it is reasonable to assume that, given the same learning ability and total number of experience instances, the effect of learning on the behavioral response will usually be largest in animals that do not have a clear preference for one or the other resource. This leads to a relationship between the innate preference and the behavioral response qualitatively similar to that depicted by the curve in Figure 1.

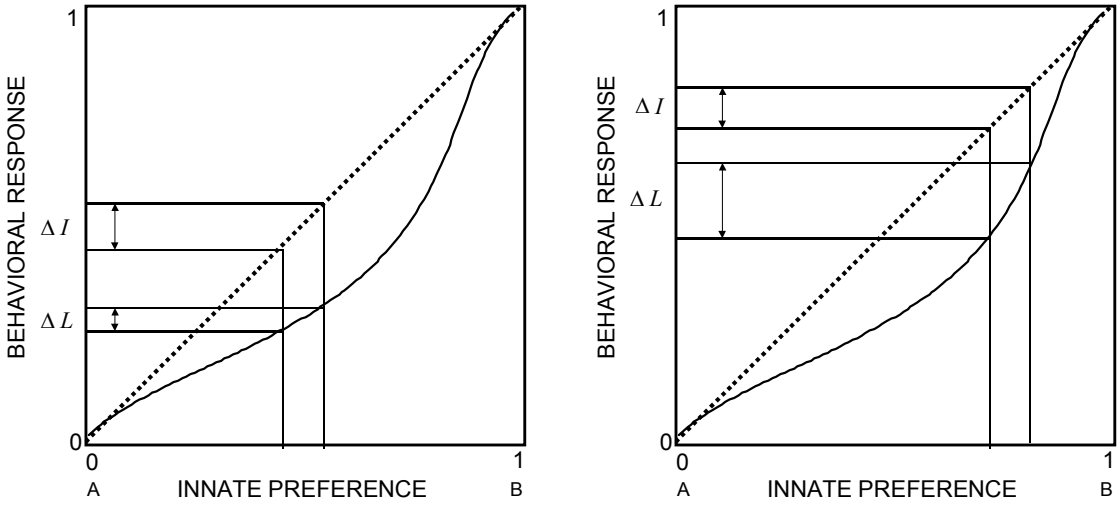

Figure 1. A model of the effect of learning on the realized behavioral response to a choice between two resources, as a function of innate preference. The preference can range from 0 (absolute preference for resource A) to 1 (absolute preference for resource B). In the absence of learning the innate preference maps linearly onto the behavioral response (diagonal dotted line). The solid line maps the innate preference onto the behavioral response when the preference is modified by negative experience with resource B (aversion learning). ΔL and ΔI denote the effect of a small difference of the innate preference on the behavioral response with and without learning, respectively. (a) Learning reduces the effect of the innate difference ($\Delta L < \Delta I$). (b) Learning magnifies the innate difference ($\Delta L > \Delta I$). For more explanation see the text.

Inspection of Figure 1 reveals that directional selection promoting the use of resource A will favor individuals with better learning ability, as learning will cause them to shift their preference away from the non-rewarding resource B. Thus, as long as the innate preference for resource A is not absolute, and unless the learning ability is very costly, learning will be favored, despite the direction of selection on the behavioral response being constant. Furthermore, depending on the innate preference, learning can either buffer (Fig. 1a) or magnify (Fig. 1b) the effect of a difference in the innate preference on the behavioral response. Assuming that fitness is linearly related to the behavioral response, learning will reduce the strength of selection on the innate preference if the population already shows an innate aversion of the non-rewarding resource (Fig. 1a). In contrast, if the population has a strong innate preference for the non-rewarding resource, learning will tend to magnify the selection differential on the innate preference, which should accelerate its evolution (Fig. 1b). To summarize, this model predicts that depending on the initial preference, learning may either slow down or facilitate the evolution of the innate preference. The range of innate preferences for which learning should facilitate versus hinder the evolution of the innate preference will depend on the exact shape of the curve, which will reflect the cognitive properties of the animal.

In order to address the interaction between the evolution of the learned and innate components of resource preference, we exposed experimental populations of *Drosophila melanogaster* to selection on both innate and learned component of oviposition substrate choice. We chose this behavioral trait for several reasons. First, in most insects oviposition site preference has a direct impact on fitness. Second, genetic variation for this trait has been demonstrated in many species (reviewed by Fox 1993). Third, *Drosophila* lay eggs in small batches throughout their adult life. The oviposition behavior is therefore performed repeatedly during the life of an individual, and each act of oviposition site choice has a small effect on fitness. Fourth, the adults feed on the oviposition substrates, which gives them a possibility to assess the substrate quality, and thus the likely consequences of the choice for offspring fitness. These characteristics make it possible for a learned component of oviposition behavior to contribute to fitness (McNeely and Singer 2001). We have recently shown that, exposed to conditions that favor learning, *D. melanogaster* easily evolve improved learning ability for oviposition substrate preference (Mery and Kawecki 2002). In the experiment described here we applied a similar approach to study the interaction between the evolution of the innate and learned component of the choice between two oviposition media, orange and pineapple.

We exposed experimental fly populations to four selection regimes, all involving the choice between these two oviposition media. In the *Innate Orange* regime the flies were selected for innate (unconditioned) preference for the orange medium. The second selection regime, *Learning Orange*, also favored flies choosing to oviposit on the orange medium, but they were additionally previously conditioned to avoid the other (i.e., pineapple) medium. The conditioning involved exposing the flies to the pineapple medium supplemented with an aversive chemical cue (quinine); selection on oviposition site preference took place 3-6 hours later. The other pair of selection regimes (respectively, *Innate Pineapple* and *Learning Pineapple*) were analogous, but they favored oviposition on the pineapple medium. The base population showed no detectable ability to respond to conditioning under these conditions (Mery and Kawecki 2002). We expected the *Innate Orange* and *Innate Pineapple* lines to evolve divergent innate preferences, but no learning ability. In contrast, our graphical model and the model by Papaj (1994) predicted that the *Learning Orange* and *Learning Pineapple* lines should evolve improved learning ability, despite the direction of selection being constant. Finally, by comparing the unconditioned preference of the lines

from the corresponding "innate" and "learning" regimes, we could test if the evolution of the innate component of preference was affected by the possibility to learn.

METHODS

Base Population

The base population originated from approximately 2000 *Drosophila melanogaster* eggs collected in the summer of 1999 in Basel (Switzerland) by setting out cups with a fruit mixture (apple and peach). The flies were subsequently maintained in uncrowded conditions on a cornmeal medium at 25°C in complete darkness and constant humidity (70-75%). The selection experiment began several months after eggs collection, which should have allowed the flies to adapt to the laboratory environment.

Experimental Evolution

The experiment consisted in four selection regimes and a fifth control regime, initially each with eight replicate lines. Every generation in each selection regime 150 adult flies (aged 14 days counted from egg) from each selection line were transferred to a cage (19 × 12 × 13 cm) and allowed to oviposit during three consecutive periods of 3 hours each, in complete darkness, 25°C, and 70 % relative humidity. During each period we offered the flies a choice between two oviposition substrates: an orange medium and a pineapple medium. These media were prepared from 100% orange or pineapple juice from concentrate and 6.6 g/l of agar. At the bottom of the cage a petri dish with 10 ml of the orange medium and another with 10 ml of the pineapple medium, each with a drop of yeast, were attached at the ends of plastic tubes (height 5 cm, diameter 6 cm). A fresh set of petri dishes with the media was provided at the beginning of the three periods; their position was randomized. Eggs laid on each medium in each period were counted; oviposition preference was scored for each period as the proportion of eggs laid on the orange medium.

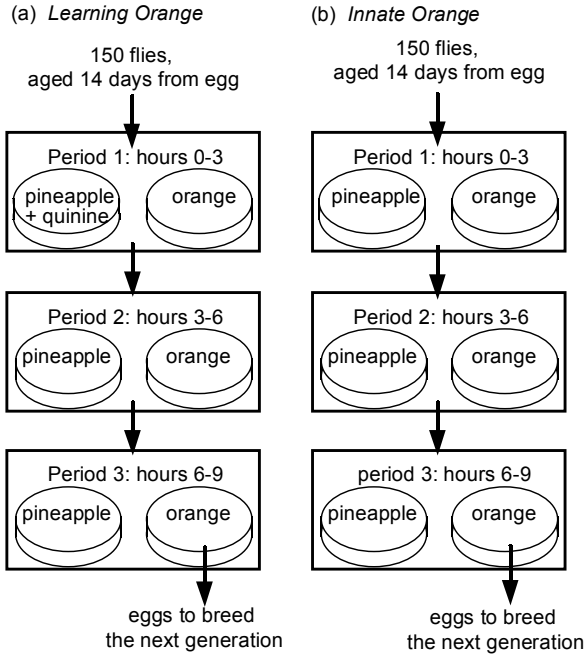

Figure 2. The design of selection regimes. (a) *Learning Orange*. (b) *Innate Orange*. Selection regimes *Learning Pineapple* and *Innate Pineapple* followed the same design except that the eggs to breed the next generation were collected from the pineapple medium, and in *Learning Pineapple* the orange medium was supplemented with quinine in period 1.

In the selection regime *Learning Orange* (Fig. 2a), the pineapple medium offered in the first 3-hour period was supplemented with 4 g/l of quinine hydrochloride. At this concentration quinine is strongly aversive when tasted by the flies, and is lethal to the larvae (F. Mery unpublished data), although several hours of exposure in the adult stage have no detectable effect on subsequent fecundity (F. Mery, unpublished data). Quinine was not added to either medium offered in periods 2 and 3. During the first period (conditioning period) the flies of this selection regime had thus an opportunity to associate the smell or taste of pineapple with the aversive taste of quinine. Their associative (aversion) learning ability would be manifested as an increased preference for the orange medium in periods 2 and 3 (test periods). Although a simpler design with a single test period would suffice to impose selection on both learned and innate components of oviposition site preference, we used two test periods to see how the response to conditioning decays with time elapsed since the end of the conditioning period. Providing fresh media after three hours also reduced the potential influence of egg overcrowding on the female motivation to oviposit. The next generation was bred from 250 eggs laid on the orange medium in period 3; in a few cases when not enough eggs were laid in period 3, we also used eggs laid on the orange medium in period 2.

The selection regime *Innate Orange* was identical to *Learning Orange* except that quinine was never added to any medium (Fig. 2b). This regime thus favored flies with innate preference for orange. Other two selection regimes, *Learning Pineapple* and *Innate Pineapple* were mirror images of *Learning Orange* and *Innate Orange*, respectively. That is, in *Learning Pineapple* the orange medium offered in period 1 was supplemented with quinine, and in both selection regimes the next generation was bred from eggs laid in period 3 on the pineapple medium. The *Control* regime involved breeding the flies from eggs laid on the orange medium in the odd-numbered generations and from eggs laid on the pineapple medium in the even-numbered generations. That is, in odd-numbered generations the *Control* regime was identical to *Innate Orange* and in even-numbered generations to *Innate Pineapple*; quinine was never added to any medium.

In all selection regimes the eggs used to breed the next generation were rinsed with water and transferred to a 250 ml bottle containing 21 ml of a standard cornmeal medium. All larvae were thus always reared on the same medium, which precluded any preferences induced by larval medium. The generation time was 14 days.

These selection regimes have been applied for 47 generations. One *Learning Orange* line was accidentally lost at generation 2. Due to technical problems (accidental insecticide poisoning in the laboratory) at generation 27 we lost 14 of the 39 lines (four lines of *Learning Pineapple*, four lines of *Innate Pineapple*, three lines of *Learning Orange*, two lines of *Innate Orange*, and one *Control* line) while the population size of some other lines was temporarily reduced (in one case to only about 20 adults). To facilitate recovery, selection regimes were suspended for generations 27-31; selection was also not applied at generations 11, 35 and 44 for other reasons. At those generations flies laid eggs on a standard cornmeal medium.

Assay of the Innate Preference and Response to Conditioning

To analyze the evolution of both components of the oviposition substrate preference, after 23 and 46 generations of selection we assayed flies from each selection regime for both the innate (unconditioned) preference, and its response to aversive conditioning. From each replicate selection line of each selection regime, as well as from each control line, we obtained three samples of 150 flies each (females + males, 14 days old counting from egg). The first sample was used to measure the innate (not conditioned) preference. This involved a design identical to that used in the *Innate Orange* and *Innate Pineapple* selection regimes. That is, the flies were transferred to a cage and allowed to oviposit for three consecutive 3-hour periods, having a choice between the orange and the pineapple medium, neither containing quinine (Fig. 2b). The second sample was conditioned to avoid pineapple in

an identical way as was done in the course of selection in the *Learning Orange* selection regime. That is, quinine was added to the pineapple medium offered in the period 1, but not periods 2 and 3 (Fig. 2a). The third sample was conditioned in an analogous way to avoid orange. Eggs laid on each medium in periods 2 and 3 were then counted. The learned component of the oviposition substrate choice would be reflected in the effect of treatment on the distribution between the media of eggs laid in periods 2 and 3.

Statistical Analysis

PROC GLM of SAS 8.02 statistical package (Littell et al. 1991) was used for all analysis. In those treatments in which quinine was added to one of the media offered in period 1, the distribution of eggs laid in period 1 simply reflected avoidance of quinine. This avoidance was almost complete from the first generation (less than 2 % of eggs were laid on the quinine-containing medium), and is of no interest here, so only eggs laid in periods 2 and 3 were included in the analysis. Realized oviposition substrate preference was measured as the proportion of eggs laid on the orange medium. These proportions were calculated separately for period 2 and 3, and $\arcsin(X^{1/2})$ transformed before the analysis (analysis of untransformed data produced virtually identical results and is not reported). We also analyzed the total number of eggs laid in the assays (pooled over period 2 and 3); large differences in the number of eggs could affect the realized preference. The results from generation 23 and 46 were analyzed separately.

Innate Preference.---The analysis of innate preference was based on the data from the treatment involving no conditioning. It addressed two questions. First, to see which selection regimes resulted in an evolutionary change of the innate preference, we used Dunnett's test to compare each selection regime with the *Control* regime. This test followed an ANOVA on data from all selection regimes, with selection regime and period as fixed factors. Replicate line was a random factor nested within selection regime, and was used as the error term for testing the effect of selection regime. This statistical model is equivalent to a repeated measures analysis of variance with replicate line as subject, period as the within-subject (repeated) effect and selection regime as a between-subject effect (Littell et al. 1991, p. 272-274). Second, to see if an opportunity to learn had an effect on the evolution of the innate preference, we compared the innate preference between the regimes *Innate Orange* and *Learning Orange*, as well as between *Innate Pineapple* and *Learning Pineapple*. These planned contrasts were done within the same ANOVA and also used replicate line (nested within selection regime) as the error term.

Response to Conditioning.---The response to conditioning was first analyzed separately for each selection regime. The arcsine-transformed proportion of eggs laid on orange was analyzed in an 3-way ANOVA using treatment (3 levels: conditioned to avoid orange, conditioned to avoid pineapple, or not conditioned) and period (2 levels) as fixed factors and replicate as a random factor. We expected that the response to conditioning should decay with time elapsed since the end of conditioning, which would be reflected in the treatment $\times$ period interaction. Treatment was tested over treatment $\times$ replicate MS, period was tested over period $\times$ replicate MS; all other effects were tested over the error term. The two treatments involving conditioning were compared with the non-conditioning treatment with Dunnett's test.

We also directly tested the hypothesis that the *Learning Pineapple* and *Learning Orange* lines show a stronger response to conditioning than the *Innate Pineapple* and *Innate Orange* lines. To do that, for each replicate line we calculated a learning score as the difference between the arcsine-transformed proportion of eggs laid on orange when conditioned to avoid pineapple and when conditioned to avoid orange (averaged over period 2 and 3). We then used the learning score as the dependent variable in a 2 × 2 factorial analysis of variance, in which the

two factors were the direction of selection regime (*Orange* versus *Pineapple*) and whether the selection regime allowed for learning (*Innate* versus *Learning*); both were treated as fixed factors. This analysis excluded the *Control* lines.

Finally, we addressed the relationship between the innate and learned components by testing if the learning ability was correlated with the innate preference among replicate lines within the selection regimes *Learning Orange* and *Learning Pineapple*. To do this we calculated the Pearson correlation coefficient between innate preference and the learning score among replicate lines within the selection regime. We did this analysis only for generation 23 because only four *Learning Pineapple* and five *Learning Orange* lines survived until generation 46.

RESULTS

Changes in the course of selection.---At generation 0 the flies of all selection regimes laid in periods 2 and 3 on average 58 % of their eggs on the orange medium. This proportion was significantly different from 50 % (t = 11.6, $P < 10^{-3}$), indicating a slight preference for the orange medium. It did not differ among the selection regimes (ANOVA, $F_{3,28}$ = 0.84, P = 0.48), suggesting no detectable response to conditioning in the base population, in agreement with our earlier results (Mery and Kawecki 2002). Figure 3. Changes of the realized preference in the course of experimental evolution. The realized preference is measured as the proportion of eggs laid on the orange medium, averaged over periods 2 and 3. Bars represent one standard error. Standard error for the control lines are not plotted for the sake of readability. Data from generations 11 and 27-31 are missing.

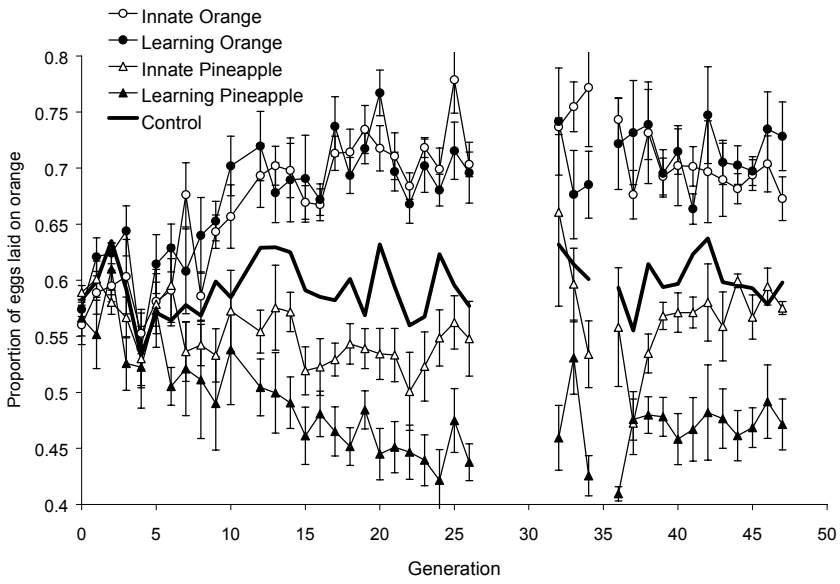

Figure 3. Changes of the realized preference in the course of experimental evolution. The realized preference is measured as the proportion of eggs laid on the orange medium, averaged over periods 2 and 3. Bars represent one standard error. Standard error for the control lines are not plotted for the sake of readability. Data from generations 11 and 27-31 are missing.

The realized oviposition substrate preference changed in the course of selection in the predicted directions (Fig. 3). Until generation 27 the proportion of eggs laid on the orange medium increased in the selection regimes *Learning Orange* and *Innate Orange*, and decreased in *Learning Pineapple* and *Innate Pineapple*, although in the *Innate Pineapple* this decrease was much slower than in the *Learning Pineapple* lines. After generation 31 no

systematic changes of the realized preference were apparent in the surviving lines of any selection regime (Fig. 3). The innate preference of the *Control* lines did not change in the course of the experiment: the proportion of eggs they laid without conditioning at generation 23 (57 %) and generation 46 (60 %) was essentially identical to that at generation 0 (58 %). No differences between *Control* populations and the base population maintained on a standard cornmeal medium were detected (Mery and Kawecki 2002).

Number of eggs.---Most replicates laid between 500 and 1000 eggs in the assays of innate preference and response to conditioning (pooled over periods 2 and 3); the average was about 720 eggs (range 175-1720). No differences among the selection regimes were detected at generation 23 ($F_{4,34}$ = 1.4, P = 0.24), but at generation 46 the *Innate Pineapple* lines laid about 50 % more eggs than the *Innate Orange* lines, with the other selection regimes and the controls ranging in between ($F_{4,21}$ = 3.1, P = 0.04). Significant variation among the replicate lines was detected at generation 23 only ($F_{34,68}$ = 2.28, P = 0.002). No effect of treatment or treatment × selection regime interaction was detected in either assay. In all selection regimes the flies laid around 20% more eggs in period 3 than in period 2. This difference probably reflects the natural tendency of fruit flies to lay more eggs in late afternoon (and therefore during period 3).

Innate preference.---The proportion of eggs laid on the orange medium in the absence of conditioning, assayed at generation 23 and 46, differed among the selection regimes (black bars in Fig. 4; $F_{4,34}$ = 25.7 and $F_{4,21}$ = 13.1, for generation 23 and 46, respectively; both P < 0.001). There was significant variation among the replicate lines at generation 23 ($F_{34,34}$ = 5.4, P < 0.001), but not at generation 46 ($F_{21,21}$ = 0.8, P = 0.7); no significant effect of period was detected. At generation 23 the *Learning Orange* and *Innate Orange* lines showed a greater innate preference for the orange medium than the *Control* populations (Dunnett's test, P < 0.05); at generation 46 this difference was significant for the *Innate Orange*, but not *Learning Orange* lines. In turn, the *Learning Pineapple* and *Innate Pineapple* lines showed at generation 23 a greater innate preference for pineapple than the *Control* lines (Dunnett's test, P < 0.05); at generation 46 this difference was detected for the *Learning Pineapple* but not the *Innate Pineapple* lines.

The *Innate Orange* lines showed a stronger innate preference for the orange medium than *Learning Orange* lines at generation 23 (contrast $F_{1,34}$ = 4.7, P = 0.04); the trend at generation 46 was in the same direction but was not significant ($F_{1,21}$ = 1.9, P = 0.19). In contrast, in both assays *Innate Pineapple* lines showed a weaker innate preference for the pineapple medium than *Learning Pineapple* lines (generation 23: $F_{1,34}$ = 6.0, P = 0.02; generation 46: $F_{1,21}$ = 8.35, P = 0.009).

Factor	*Innate Orange*	*Innate Pineapple*	*Learning Orange*	*Learning Pineapple*	*Control*
Generation 23					
Treatment	$F_{2, 14} = 0.33$	$F_{2, 14} = 2.43$	$F_{2, 12} = 3.03$†	$F_{2, 14} = 43.0$ ***	$F_{2, 14} = 0.93$
Period	$F_{1, 7} = 0.00$	$F_{1, 7} = 0.18$	$F_{1, 6} = 0.26$	$F_{1, 7} = 4.80$†	$F_{1, 7} = 2.32$
Replicate	$F_{7, 14} = 0.88$	$F_{7, 14} = 8.22$ **	$F_{6, 12} = 2.97$†	$F_{7, 14} = 14.0$ **	$F_{7, 14} = 2.51$†
Treatment × period	$F_{2, 14} = 1.09$	$F_{2, 14} = 0.29$	$F_{2, 12} = 1.42$	$F_{2, 14} = 7.20$ **	$F_{2, 14} = 2.27$
Treatment × replicate	$F_{14, 14} = 2.02$	$F_{14, 14} = 0.81$	$F_{12, 12} = 0.69$	$F_{14, 14} = 1.69$	$F_{14, 14} = 2.46$†
Replicate × period	$F_{7, 14} = 0.85$	$F_{7, 14} = 0.14$	$F_{6, 12} = 1.24$	$F_{7, 14} = 1.14$	$F_{7, 14} = 0.70$
Generation 46					
Treatment	$F_{2, 10} = 1.42$	$F_{2, 6} = 2.97$	$F_{2, 8} = 5.04$*	$F_{2, 6} = 26.8$**	$F_{2, 12} = 0.33$
Period	$F_{1, 5} = 3.34$	$F_{1, 3} = 0.21$	$F_{1, 4} = 1.08$	$F_{1, 3} = 1.57$	$F_{1, 6} = 3.39$
Replicate	$F_{5, 10} = 3.21$†	$F_{3, 6} = 1.49$	$F_{4, 8} = 1.39$	$F_{3, 6} = 0.93$	$F_{6, 12} = 1.02$
Treatment × period	$F_{2, 10} = 4.73$	$F_{2, 6} = 0.50$	$F_{2, 8} = 1.34$	$F_{2, 6} = 0.07$	$F_{2, 12} = 0.82$
Treatment × replicate	$F_{10, 10} = 2.22$	$F_{6, 6} = 0.43$	$F_{8, 8} = 1.58$	$F_{6, 6} = 0.42$	$F_{12,12} = 0.66$
Replicate × period	$F_{5, 10} = 2.52$	$F_{3, 6} = 2.53$	$F_{4, 8} = 0.96$	$F_{3, 6} = 0.42$	$F_{6, 12} = 0.46$

†0.1 > P > 0.05; P < *0.05; **P < 0.01; ***P < 0.001

Table 1. Results of the ANOVA on the proportion of eggs laid on the orange medium with and without conditioning (assays at generation 23 and 46). The analysis has been done separately for the two assays and for each selection regime. Treatment was tested over treatment × replicate MS; period was tested over period × replicate MS; other factors were tested over the residual MS.

Response to Conditioning.---The analysis of response to conditioning is summarized in Table 1. There was significant variation of the realized preference among the replicate lines of *Innate Pineapple* at generation 23, and there was a marginally significant effect of replicate and treatment × replicate interaction in the *Control* lines (Table 1). No other significant effect was detected for these two selection regimes, and no effect was detected for *Innate Orange*. Specifically, we detected no response to conditioning by the *Innate Orange*, *Innate Pineapple* and *Control* lines (Fig. 4).

In contrast, the oviposition preference of the *Learning Pineapple* populations was strongly affected by conditioning both at generation 23 and at generation 46 (Fig. 4). This response was, however, strongly asymmetric: *Learning Pineapple* lines responded when conditioned to avoid pineapple (Dunnett's test, P < 0.002 in both assays), but not when conditioned to avoid orange (P > 0.3 at generation 23 and 46). Additionally, the flies

conditioned to avoid orange laid a greater proportion of eggs on the orange medium in period 3 than in period 2

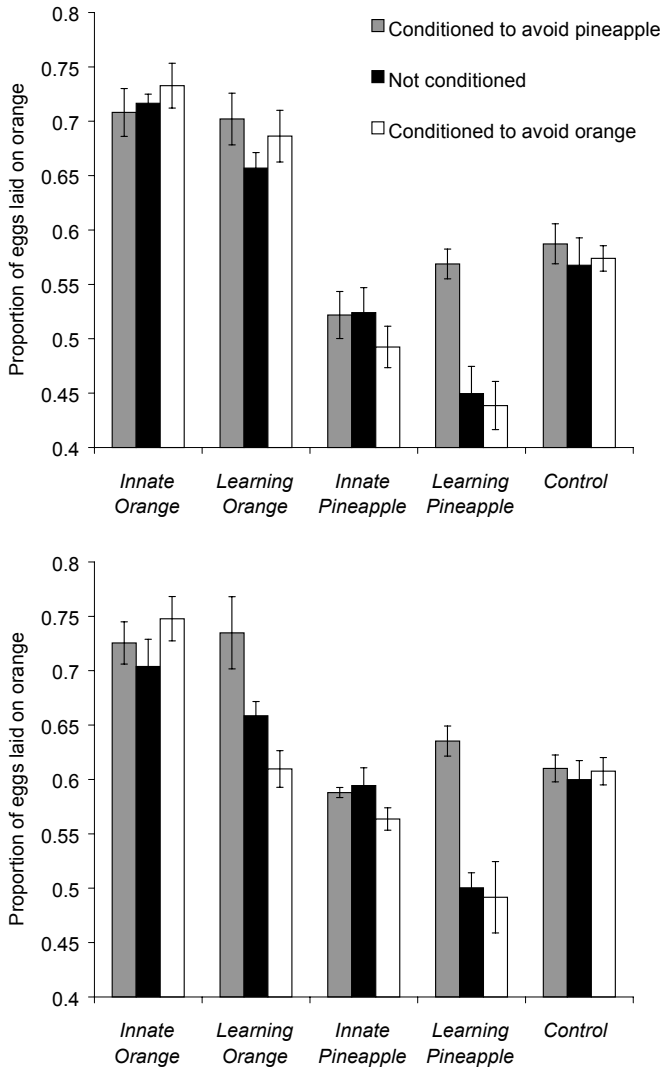

Figure 4. The innate preference and the response to conditioning assayed at generation 23 (a) and 46 (b). "Conditioned to avoid pineapple" means that quinine was present in the pineapple medium offered in period 1. For the plot the proportion of eggs laid on the orange medium was averaged over periods 2 and 3.

(paired *t*-test, $t = 3.61$, *d.f.* = 7, $P = 0.009$). When only eggs laid in period 2 were included in the analysis, Dunnett's test detected a significant response to conditioning in either direction ($P < 0.01$ for both directions and both generations). This difference is reflected in the treatment × period interaction, but it also contributed to the main effect of period. No difference between period 2 and 3 was detected in *Learning Pineapple* for the other two treatments (both $P > 0.7$). The *Learning Orange* lines were affected by conditioning only at generation 46 (Fig. 4, Table 1). The response was less asymmetric than that of *Learning Pineapple*, and Dunnett's test could not detect any difference of oviposition preference between flies conditioned in either direction and the not conditioned treatment ($P > 0.2$).

The 2 × 2 analysis of variance on the learning score confirmed that flies from the two selection regimes involving learning (*Learning Orange* and *Learning Pineapple*) showed better learning compared to the two *Innate* selection

regimes ($F_{1,27}$ = 8.1, P = 0.008 and $F_{1,15}$ = 23.0, P = 0.0002 at generation 23 and 46, respectively). This analysis also indicated a significant effect of the direction of selection at generation 23 ($F_{1,27}$ = 11.0, P = 0.003), reflecting the fact that the *Learning Pineapple* lines showed a clear response to conditioning while the *Learning Orange* lines did not, but also that the *Innate Pineapple* lines tended to show a positive learning score while the *Innate Orange* showed no such trend (Fig. 4a). This difference disappeared in generation 46 ($F_{1,15}$ = 1.0, P = 0.3). The interaction between the direction of selection and the opportunity for learning was not significant in either assay (both P > 0.2).

At generation 23 we observed a marginally significant positive correlation among replicate lines of the *Learning Pineapple* selection regime between the learning score and innate preference for pineapple (Fig. 5 top, Pearson's r = 0.69, P = 0.06). The correlation was similarly strong when calculated separately for period 2 and 3 (r = 0.67, P = 0.07, and r = 0.69, P = 0.06, respectively). It was also virtually identical when calculated with the original (untransformed) data, indicating that it is not an artifact of scale. Among the replicate lines of *Learning Orange* those with a high innate preference for the orange medium tended to show a low learning score (Fig. 5 bottom, Pearson's r = –0.61, P = 0.15, the learning score and innate preference averaged over the two periods).

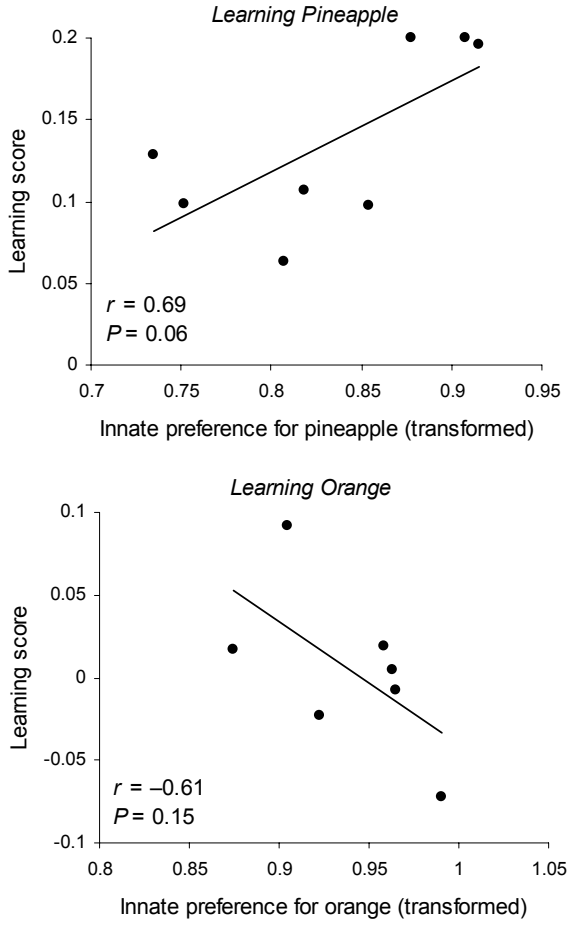

Figure 5. The relationship between the innate preference for the favored medium and learning ability among the *Learning Pineapple* lines (top) and among the *Learning Orange* lines (bottom), measured at generation 23. The learning score was calculated as the difference between oviposition preference when conditioned to avoid pineapple and when conditioned to avoid orange. Both scales are angularly-transformed. The lines are least-square regressions.

DISCUSSION

By exposing populations of *D. melanogaster* to selection either on only innate oviposition site preference, or on both innate and learned components of this behavior, we aimed to address two main questions. First, will learning ability also evolve in a stable environment, where a fixed behavioral response is favored? Second, does an evolutionary change of learning ability affect the evolution of the innate component? The control for the latter question is provided by regimes only selecting on the innate component of preference (*Innate Orange* and *Innate Pineapple*); we discuss their response first.

The Evolution of Innate Preference

Selection regimes *Innate Orange* and *Innate Pineapple* involved selection on the innate component of oviposition substrate preference. Within 23 generations the *Innate Orange* lines increased their innate preference for the orange medium from 58 % to 72 % (Fig. 3). In contrast, the *Innate Pineapple* lines showed only a weak trend to change their innate preference towards laying more eggs on the pineapple medium (from 42 % to 47 % within 23 generations). This trend disappeared following the suspension of selection in generations 27-31, and the surviving *Innate Pineapple* lines did not show a consistent response after selection was resumed. The surviving *Innate Orange* lines also showed no further response to selection after generation 31, but the evolutionary change that occurred before generation 27 did not become reversed (Fig. 3).

Oviposition substrate preference and its evolutionary aspects have been extensively studied in herbivorous insects (Futuyma and Moreno 1988; Radtkey and Singer 1995; Thompson 1998; Scriber 2002; Nosil 2002). Although evolutionary changes of host preference in nature have been documented (Singer 1993), experimental studies have often failed to obtain a response to selection on preference (Wasserman 1986, Thompson 1993). Thompson (1993; Wehling and Thompson 1997) proposed three possible mechanisms that may be responsible for evolutionary conservatism of oviposition preference: genetic constraints, weak selection, and gene flow. Our experimental design precluded gene flow. Furthermore, selection was rather strong: other things being equal, the contribution of a female fly to the next generation was proportional to the number of eggs laid on the correct medium. The adult population size of 150 individuals made it unlikely for drift to be able to overcome the effects of selection during the first half of the experiment (until generation 27), although drift is likely to have played a role during the bottleneck caused by the insecticide poisoning at generation 27. Therefore, broadly defined genetic constraints remain the most plausible explanation for the poor response of the *Innate Pineapple* lines to selection. However, genetic variation for a greater innate preference for the pineapple medium did exist in our base population, as the *Learning Pineapple* lines did evolve a stronger innate preference for this medium. Selection under the *Innate Pineapple* selection regime may have been opposed by possible deleterious pleiotropic effects of alleles for pineapple preference. Although we cannot exclude this possibility, it would be difficult to imagine why such pleiotropy should not affect the *Learning Pineapple* lines as well. Another possibility is that the greater innate preference of some individuals for pineapple may have been overridden by a tendency to oviposit where other females have oviposited. This scenario is discussed below in the subsection on the interaction between innate and learned components of preference.

The Evolution of Learning Ability

In both assays (generation 23 and 46) flies from populations subject to the *Learning Pineapple* selection regime showed an ability to respond to conditioning. The *Learning Orange* flies also showed a response to conditioning at generation 46, although not at generation 23. In contrast, the *Innate Pineapple*, *Innate Orange*, and *Control* lines did not respond to conditioning in either assay. This indicates that *Learning Pineapple* and *Learning*

Orange have evolved an improved ability for aversion learning. In contrast to the usual argument that learning is only favored in temporally varying environments (Dukas 1998), here learning ability has evolved under directional selection regimes that always favored the same response. This follows our argument discussed in the introduction, and the prediction of a model by Papaj (1994). Papaj predicted that the selective advantage of learning ability under invariable directional selection is likely to be transient – the advantage of learning disappears when the innate preference for the "good" medium has approached 100 %. However, after almost 50 generations of selection, our flies were still far from this limit, and so learning may still have helped to increase the proportion of eggs laid on the favored medium. Furthermore, slowing down of the response suggests that a 100 % innate preference may be difficult or impossible to reach, in which case the advantage of learning may be permanent.

On the other hand, in both assays (generation 23 and 46) the ability to learn in the *Learning Pineapple* lines was much more strongly manifested when they were conditioned to avoid pineapple than when they were conditioned to avoid orange, even though in this selection regime the flies were conditioned every generation to avoid orange. This strong asymmetry of the response to conditioning was specific to the *Learning Pineapple* selection regime. There was only a weak tendency for such asymmetry in the *Learning Orange* lines at generation 46, and it was not observed at all in another set of lines, which originated from the same base population and had been selected for improved response to conditioning in both directions (Mery and Kawecki 2002). Such an asymmetry would have been easier to explain if the flies had shown a strong innate preference for pineapple. If this had been the case, the flies would have avoided the orange medium during conditioning (in period 1) and would thus have had little opportunity to associate it with quinine. However, in the absence of conditioning the *Learning Pineapple* lines laid on average about 45 % of eggs on the orange medium, which means that they should have had enough contact with the quinine-containing orange while being conditioned to avoid orange.

It should also be noted that the observed response to conditioning is based on the proportion of eggs laid on the two media during 6 hours (i.e., two 3-hour periods) after the end of the conditioning period. Therefore, it is possible that the *Learning Pineapple* flies conditioned to avoid orange show a strong response immediately after conditioning, but quickly lose the response when they realize that the orange medium does not contain quinine any more. This process of erasing an old learned response through new experience is referred to as memory extinction (Bouton 1994). This idea is supported by the response observed within 3 hours (period 2) and between 3 and 6 hours (period 3) after conditioning. The proportion of eggs laid on the orange medium by *Learning Pineapple* flies conditioned to avoid orange was significantly smaller in period 2 than period 3, and a response to conditioning could only be detected in period 2. In contrast, the realized preference did not differ between the two periods for *Learning Pineapple* flies conditioned to avoid pineapple, as well as for those not conditioned. It is, however, not clear why the *Learning Pineapple* lines should show a faster memory extinction when conditioned to avoid orange than when conditioned to avoid pineapple. Their selection regime favored flies that were not prone to memory extinction, i.e., continued to avoid the orange medium 3 to 6 hours after the termination of conditioning. Thus, the asymmetry of the response of *Learning* Pineapple lines to conditioning remains unexplained.

Despite these open questions, our results seem to be the first to demonstrate directly evolution of improved learning ability in a constant environment, in which the same behavioral response is always optimal. They suggest the need for some re-thinking of the evolutionary theory of learning ability.

The Evolutionary Interaction Between the Innate and Learned Component of Preference

Comparison of the innate preference between the selection regimes *Learning Orange* and *Innate Orange*, as well as between *Learning Pineapple* and *Innate Pineapple*, indicates that the opportunity to learn has affected the

evolution of innate preference. Under selection favoring oviposition on the orange medium learning has slowed down the evolution of the innate preference: *Innate Orange* lines had a stronger innate preference for orange than *Learning Orange* lines. In contrast, under selection for oviposition on pineapple learning has accelerated the evolution of the innate preference: *Learning Pineapple* lines had a stronger innate preference for pineapple than *Innate Pineapple* lines. This synergism between the evolution of the innate preference and learning ability under the *Learning Pineapple* selection regime is also apparent in the pattern of variation among replicate selection lines. Across the replicate lines the learning score was positively correlated with the innate preference for pineapple, i.e., the proportion of eggs laid on the pineapple medium in the absence of conditioning (Fig. 5 top). Despite involving only eight points, this correlation is very close to significance at the conventional level ($p = 0.06$). In contrast, across replicate *Learning Orange* lines the correlation between innate preference for the favored medium (i.e., orange) and the learning score tended to be negative (Fig. 5 bottom), although far from significant. Taken at its face value, this correlation is consistent the idea that learning has hampered the evolution of innate preference under the *Learning Orange* selection regime.

These results are consistent with the graphical model we proposed in the introduction (Fig. 1). This model suggests that, depending on the initial innate preference for the favored resource, learning may either hinder or facilitate the evolution of the innate component of preference. At the start of the selection experiment the flies showed a weak but significant innate preference for orange over pineapple (58 % versus 42 % of eggs). Thus, selection favoring oviposition on the orange medium, for which the innate preference was already high at the start, might have created conditions resembling the scenario described in figure 1a. In that scenario learning reduces the effective selection differential on the innate component of preference. In contrast, selecting for increased oviposition on the initially less-preferred pineapple medium may have corresponded to the scenario described in figure 1b, in which learning magnifies the selection differential on the innate preference. However, any conclusions concerning the validity of the model, even for our study system, would be premature. In particular, the relationship between the innate and the learned component of preference we assumed in the model requires empirical verification. Also, the model does not explain the difference in response to selection between the selection regimes *Innate Pineapple* and *Innate Orange*.

An alternative explanation invokes a potential additional selection pressure due to the exposure to quinine during the conditioning period (period 1) in the “learning” selection regimes. The exposure to quinine may have had some detrimental effect on the number or quality of eggs laid in period 3 six hours later; these were the eggs used to breed the next generation. If so, *Learning Pineapple* flies would have been under stronger selection to avoid the orange medium than *Innate Pineapple*, because by avoiding the orange medium in period 1 the former would also avoid contact with quinine. However, the above argument would predict that replicate lines of the *Learning Pineapple* selection regime that have evolved a stronger innate preference for pineapple would have been under weaker selection for learning ability. Furthermore, the same argument would hold for the selection to avoid pineapple in the *Learning Orange* lines, yet those lines evolved weaker innate preference for orange than the *Innate Orange* lines. One could still argue that *Learning Orange* flies were from the beginning less prone to this effect, as they experienced quinine in the pineapple medium, which from the start was the less preferred one. This would, however, not explain why the *Learning Orange* lines evolved a weaker innate preference for orange than the *Innate Orange*. Finally, we have not observed any reduction of fertility or survival due to the exposure to quinine in the first generations of selection. Nonetheless, we cannot exclude that the exposure to quinine did contribute to the effective selection pressure on the innate components of oviposition substrate preference.

The outcome of selection may also have been affected by an interaction among innate preference, learning, and the flies' tendency for gregariousness. *D. melanogaster* females like to oviposit in sites already containing conspecific eggs (Delsolar and Godoy 1971; Rockwell and Grossfield 1978). Thus, the proportion of eggs laid by an individual on each medium will depend on its innate preference and possibly learning, but also on the oviposition decisions of other individuals. This gregarious tendency might override the innate preference of an individual for pineapple if the rest of the population shows a strong preference for orange. If so, a rare allele for increased innate preference for pineapple would be effectively underexpressed, and the evolution of increased innate preference for pineapple would be constrained. This constraint would be alleviated if the rest of the population increased oviposition on pineapple in response to conditioning. This would thus be another mechanism in which learning could facilitate the evolution of innate preference. It would potentially explain both the weak response of the *Innate Pineapple* lines to selection on the innate component of preference, as well as the fast evolution of the innate component of preference in the *Learning Pineapple* lines. However, it is unclear whether the moderate preference for orange shown by all lines at the onset of the experimental evolution (58 % of eggs laid on the orange medium) was strong enough for the gregarious tendency to have a non-negligible effect. More work is needed to examine the relative importance for oviposition choice of individual innate preference, own experience and gregariousness.

CONCLUSION

The work described above is, to our knowledge, the first to use experimental evolution to address the interaction between the learned and innate components of behavior in the course of evolution. This approach allowed us to show that learning ability can evolve even if the same behavioral response is favored every generation. We were also able to provide the first direct evidence for a synergistic effect between learning and the evolution of the innate component of behavior. Finally, the results suggest that the outcome of the evolutionary interaction between the innate and learned components depends on the direction of selection. These results indicate that experimental evolution in model laboratory systems offers a promising approach to study the evolution of the innate and learned components of behavior, particularly in the context of diet, host, or habitat choice.

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-Alors c'est grave…" (Audiard M.)

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PUBLICATIONS

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- **Mery F**, Kawecki TJ 2002 “Experimental evolution of learning ability in fruit flies” *Proc. Natl Acad Sci USA* 99: 14274-14279

In preparation or submitted

- **Mery, F**. and Kawecki, T. J. “A fitness cost of learning ability in Drosophila melanogaster” In preparation for *Proc. Natl Acad Sci USA*
- **Mery, F**. and Kawecki, T. J. “An induced fitness cost of learning in Drosophila melanogaster” In preparation for *Animal Behaviour*
- **Mery, F**. and Kawecki, T. J. “Evolutionary interactions between the innate and learned components of behavior: experimental evolution of oviposition substrate preference in Drosophila melanogaster” In preparation for *Evolution*
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- **2003**- Invited talk : ‘Experimental approach to the costs and benefits of animal learning’, Université Pierre et Marie Curie, Paris, France, (January).
- **2002**- Talk : “Evolution des capacités d’apprentissage chez la drosophile” XXIVème réunion du Groupe de Génétique et Biologie des Populations, Montpellier, France, (August).
- **2002**- Invited talk :“Approche expérimentale des couts et bénefices liés aux capacités d’apprentissage ” Séminaires du Groupe d’Evolution de la Faculté des Sciences de Montpellier II, Université de Montpellier, France, (May).
- **2002**- Invited talk : “Experimental evolution of learning ability in *Drosophila melanogaster* - How learning affects evolution ?” Institute for Cell, Animal and Population Biology, University of Edinburgh, Edinburgh, United-Kingdom (April).
- **2002**- Invited talk : “Experimental evolution of learning ability in fruit flies” Institute of Genetics and Neurobiology, University of Würzburg, Würzburg, Germany (March).
- **2001**- Invited talk : “Experimental evolution of learning ability *Drosophila melanogaster*” Zoologisches Institut Bern-University of Bern, Switzerland (November).

- **2001**- Poster presentation : “Experimental evolution of learning ability in fruit flies-How learning affects evolution of the innate behaviour ?”. 8th Congress of The European Society for Evolutionary Biology, Aarhus, Denmark (August).
- **2001**- Poster presentation : “Experimental evolution of learning ability in *Drosophila melanogaster*” Animal behaviour meeting, Zurich, Switerland (June).
- **2001**- Invited talk: “How learning affects evolution ?” Centre d’Ecologie Fonctionelle et Evolutive, Montpellier, France (May).
- **1999**- Talk : “Male female conflicts and the management of giant sperm: study on *Drosophila bifurca*”. VII Congress of the European Society for the Study of Evolution. Barcelona, Spain (August).
- **1998**- Poster presentation : “ Evolution du gignatisme des spermatozoides chez *Drosophila bifurca*” XXème réunion du Groupe de Génétique et Biologie des Populations, Lille, France (August).

SCIENTIFIC EVALUATION AND ORGANISATION

Referee: Journal of Evolutionary Biology (2), Ecology Letters (1)

Organizer

- 2000-2003 : founder and organizer of seminar series “Interaction Seminars” in Evolutionary Biology (Web page: http://www.unifr.ch/biol/ecology/seminars/interaction_seminar.html)

20-21 February 2003: Organizer of an international workshop “Evolutionary Biology of Learning” at the university of Fribourg. (Web page: http://www.unifr.ch/biol/ecology/learningworkshop)